

This electronic thesis or dissertation has been downloaded from the King's Research Portal at <https://kclpure.kcl.ac.uk/portal/>



THE ANGIOGENIC RESPONSE OF HUMAN DENTAL PULP TO ORTHODONTIC FORCE APPLICATION

Willson, Timarah Grace

Awarding institution:
King's College London

The copyright of this thesis rests with the author and no quotation from it or information derived from it may be published without proper acknowledgement.

END USER LICENCE AGREEMENT



Unless another licence is stated on the immediately following page this work is licensed

under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International

licence. <https://creativecommons.org/licenses/by-nc-nd/4.0/>

You are free to copy, distribute and transmit the work

Under the following conditions:

- Attribution: You must attribute the work in the manner specified by the author (but not in any way that suggests that they endorse you or your use of the work).
- Non Commercial: You may not use this work for commercial purposes.
- No Derivative Works - You may not alter, transform, or build upon this work.

Any of these conditions can be waived if you receive permission from the author. Your fair dealings and other rights are in no way affected by the above.

Take down policy

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

THE ANGIOGENIC RESPONSE OF HUMAN DENTAL PULP TO ORTHODONTIC FORCE APPLICATION

By

TIMARAH GRACE WILLSON

A thesis submitted in partial fulfilment of the requirements for the
degree of

DOCTOR OF PHILOSOPHY

KING'S COLLEGE LONDON

2016

- 1 -

ACKNOWLEDGMENTS

I would like to thank my supervisor Dr Kathryn Derringer for all of the help and support she's given me over the years. Thank you for all of your time and the sacrifices you've made in order to help me acquire the samples I needed to perform these experiments. Thank you for performing all of the orthodontic treatment, tooth extraction and pulp isolation. Thank you for providing an interesting question that has been a challenge to attempt to answer.

To my second supervisor Prof. Gordon Proctor, I am eternally indebted to you for all of your advice, direction and unwavering support. Thank you for guiding me through this project with all of its challenges and for giving me a home in the Salivary Research Group. Being a part of your lab group was an honour and an experience I will treasure for many years to come.

I would like to thank the King's Dental Institute Research Studentship Programme, King's College London and the Guy's and St Thomas' NHS Foundation Trust and King's College London's comprehensive Biomedical Research Centre for funding this project and supporting this area of translational research.

A huge thank you to Dr A.N.A. (Nora) Donaldson for all of the time she has dedicated to the statistical analysis of the data. Thank you for the hours of faxing, emailing and phone calls; for the advice and the discussions on which approaches to take. I am so grateful that you have been a part of this project.

Thank you to Prof. Agi Grigoriadis for giving me space to work in your lab for the first six months of my project and for providing the MG-63 osteosarcoma cells.

Thank you to Dr Gilles Bluteau and Dr Nick Silver for your help with designing primers and advice on optimising experimental protocols.

To my friends, Dr Emanuele Cotroneo and Ms Rashida Pramanik, thank you so much for all of your advice and the support you've given me during this PhD! Thank you for the lunches, late nights and the weekends spent thrashing out problems and discussing angles at which to approach the project; for reading through drafts for grants, conference abstracts and papers; for being a shoulder to cry on and constant source of laughter. You have been my family for the time I was in London and will always hold a very special place in my heart.

Finally, I would like to thank my family, Papa Bear, Lady Duck and Spud, for all of their love, support and encouragement throughout these years. You have lifted and carried me at times when I didn't think there was a way forward. Your unwavering faith and belief in me inspired me to take on this challenge and to never give up. I am so proud to be called your daughter, sister and friend. I love you five bob, Tadpole x

I dedicate the completion of this work to the memory of my father

Malcolm Gordon Willson

1944 –2013

Abstract

In order to characterise the angiogenic response of human dental pulps to orthodontic force application, transcriptomic and proteomic changes were investigated relative to three early time-points of routine clinical treatment in contralateral pairs of treated and control teeth. It was hypothesised that varied intensities of hypoxic exposure (intermittent, repeated intermittent and chronic) would result in distinct angiogenic responses within untreated pulp tissues. It was further hypothesised that angiogenic responses of pulps exposed to early time-points of orthodontic treatment would correlate with those of tissues exposed to varied hypoxic intensities; and changes would be associated with known stages of clinical treatment, namely: initial, lag, acceleration and linear phases of orthodontic tooth movement.

Prior to performing all experimentation, methodologies for handling of rare clinical samples were established. Protocols for sample storage, homogenisation, RNA_{total} isolation, quantification, purification and amplification were optimised; a normalising factor created from the mean expression of reference genes RPL13A and UBC was found to be most appropriate for RT-qPCR analysis of human dental pulps under these experimental conditions.

Orthodontic force application resulted in a potential angiogenic or putative inflammatory response at all treatment time-points; and altered gene expression associated with hypoxia is evident in pulps following 2 weeks of force application. The response to each defined hypoxic intensity was unique; and the associated angiogenic response correlated with the duration of hypoxic exposure. Reoxygenation following hypoxia was linked to the putative inflammatory response of pulps during orthodontic treatment.

Results showed that the experimental potential of a single clinical sample can be amplified significantly, dependent upon the selection of specific experimental protocols; thus eliminating the need for pooling samples or using cell lines which are far removed from true physiological conditions. Both angiogenesis and hypoxia appear to have a significant role in response of human dental pulps to routine orthodontic treatment.

Table of Contents

Abstract.....	- 4 -
Declaration of Word Count.....	- 17 -
List of Presentations.....	- 18 -
List of Abbreviations.....	- 20 -
List of Figures	- 29 -
List of Tables	- 31 -
Aims of the Thesis	- 37 -
Chapter I Introduction	- 38 -
Introduction.....	- 39 -
<i>1.1.1 Origins of the Dental Pulp – An Overview of Tooth Development.....</i>	<i>- 40 -</i>
<i>1.1.2 Anatomy of the Dental Pulp</i>	<i>- 47 -</i>
<i>1.1.2.1 Dental Pulp Morphology</i>	<i>- 47 -</i>
<i>1.1.2.2 Dental Pulp Vasculature</i>	<i>- 49 -</i>
<i>1.1.3 Orthodontic Tooth Movement</i>	<i>- 51 -</i>
<i>1.1.3.1 Mechanisms of Orthodontic Tooth Movement.....</i>	<i>- 51 -</i>
<i>1.1.3.2 Response of the Human Dental Pulp to Orthodontic Tooth Movement.....</i>	<i>- 53 -</i>
<i>1.1.4 Angiogenesis</i>	<i>- 54 -</i>
<i>1.1.4.1 Intussusceptive Angiogenesis</i>	<i>- 54 -</i>
<i>1.1.4.2 Sprouting Angiogenesis.....</i>	<i>- 56 -</i>
<i>1.1.5 Hypoxia and Angiogenesis</i>	<i>- 58 -</i>

1.1.6	<i>Hypoxic and Angiogenic Growth Factors in the Human Dental Pulp</i>	- 59 -
Chapter II Materials and Methods		- 62 -
MATERIALS.....		- 63 -
2.1	Human Dental Pulp Tissue	- 63 -
METHODS		- 63 -
2.2	Orthodontic Force Application and Dental Pulp Isolation	- 63 -
2.3	RNA Isolation	- 64 -
2.4	RNA Quantification	- 65 -
2.5	Reverse Transcription	- 65 -
2.6	Quantitative real-time reverse-transcription Polymerase Chain Reaction (RT-qPCR).....	- 66 -
2.7	Statistical Analysis of RT-qPCR Data.....	- 67 -
Chapter III Optimising Protocols for the Analysis of Human Dental Pulp at the Molecular Level		- 68 -
3.1	Introduction.....	- 69 -
3.2	Aims	- 72 -
3.3	Materials and Methods.....	- 73 -
MATERIALS.....		- 73 -
3.3.1	<i>MG-63 Osteosarcoma Cells</i>	- 73 -
3.3.2	<i>Human Dental Pulps</i>	- 73 -
METHODS		- 73 -
3.3.3	<i>Cell Culture</i>	- 73 -

3.3.4	<i>Orthodontic Force Application and Dental Pulp Isolation</i>	<i>- 74 -</i>
3.3.5	<i>Pulp Homogenisation for RNA Isolation</i>	<i>- 75 -</i>
3.3.5.1	<i>Mortar and Pestle</i>	<i>- 75 -</i>
3.3.5.2	<i>Passing Tissue through a Needle</i>	<i>- 76 -</i>
3.3.5.3	<i>FastPrep®-24 Instrument</i>	<i>- 76 -</i>
3.3.5.4	<i>Glass Dounce Tissue Homogeniser</i>	<i>- 76 -</i>
3.3.6	<i>RNA Isolation Techniques.....</i>	<i>- 77 -</i>
3.3.6.1	<i>Phenol:Chloroform & DNase Treatment.....</i>	<i>- 77 -</i>
3.3.6.2	<i>RNEasy Mini Kit & DNase Treatment.....</i>	<i>- 78 -</i>
3.3.6.3	<i>Phenol:Chloroform & RNEasy Mini Kit</i>	<i>- 79 -</i>
3.3.6.4	<i>RNA Isolation from MG-63 Osteosarcoma Cells</i>	<i>- 79 -</i>
3.3.7	<i>RNA Quantification Techniques.....</i>	<i>- 80 -</i>
3.3.7.1	<i>Spectrophotometry</i>	<i>- 80 -</i>
3.3.7.2	<i>NanoDrop Spectrophotometry.....</i>	<i>- 80 -</i>
3.3.7.3	<i>Ribogreen® RNA Assay Kit.....</i>	<i>- 81 -</i>
3.3.8	<i>Assessment of RNA Integrity – Bioanalysis of RNA.....</i>	<i>- 81 -</i>
3.3.9	<i>Reverse-Transcription Techniques</i>	<i>- 81 -</i>
3.3.9.1	<i>Moloney Murine Leukaemia Virus Reverse Transcriptase</i>	<i>- 81 -</i>
<u>3.3.9.1.a</u>	<u><i>Method A</i></u>	<u><i>- 81 -</i></u>
<u>3.3.9.1.b</u>	<u><i>Method B</i></u>	<u><i>- 82 -</i></u>
<u>3.3.9.1.c</u>	<u><i>Method C</i></u>	<u><i>- 82 -</i></u>
3.3.9.2	<i>iScript</i>	<i>- 83 -</i>
3.3.9.3	<i>WT-Ovation™ Pico RNA Amplification.....</i>	<i>- 83 -</i>
3.3.10	<i>Selection of Candidate Reference Genes and Angiogenic Growth Factors for mRNA Expression Analysis.....</i>	<i>- 84 -</i>
3.3.11	<i>Primer Design</i>	<i>- 85 -</i>

3.3.11.1	<i>Primers for RT-PCR of Angiogenic Growth Factors and the Reference Gene GAPDH.....</i>	- 86 -
3.3.11.2	<i>Primers for RT-qPCR of Angiogenic Growth Factors.....</i>	- 88 -
3.3.11.3	<i>Primers for RT-qPCR of Reference Genes.....</i>	- 90 -
3.3.12	<i>Reverse-Transcription PCR (RT-PCR).....</i>	- 93 -
3.3.13	<i>Reverse-Transcription Quantitative Real-Time PCR (RT-qPCR) Analysis of mRNA Expression.....</i>	- 94 -
3.3.14	<i>Gel Electrophoresis.....</i>	- 95 -
3.3.15	<i>Statistical Analysis of RT-qPCR Data.....</i>	- 96 -
3.3.15.1	<i>Reference Gene Expression Stability Analysis</i>	- 96 -
3.3.15.2	<i>Gene Expression Analysis for Angiogenic Growth Factors</i>	- 96 -
3.4	<i>Results.....</i>	- 98 -
3.4.1	<i>Sample Storage and RNA Preservation</i>	- 98 -
3.4.2	<i>RNA Isolation, Yield and Sample Purity.....</i>	- 98 -
3.4.2.1	<i>Optimisation of Tissue Homogenisation and RNA Isolation Protocols</i>	- 98 -
3.4.2.2	<i>Optimisation of RNA Quantification Protocols.....</i>	- 102 -
3.4.3	<i>Reverse-Transcription for RT-PCR and RT-qPCR.....</i>	- 104 -
3.4.3.1	<i>Optimisation of Reverse-Transcription Protocols for RT-PCR</i>	- 104 -
3.4.3.2	<i>Optimisation of Reverse-Transcription Protocols for RT-qPCR</i>	- 105 -
3.4.4	<i>Normalising to total RNA and Distribution of Reference Gene Expression.....</i>	- 107 -
3.4.5	<i>Analysis of Reference Gene Expression Stability – BestKeeper©</i>	- 109 -
3.4.6	<i>Analysis of Reference Gene Expression Stability – geNorm and NormFinder.....</i>	- 112 -
3.4.7	<i>Expression of Angiogenic Growth Factors in Human Dental Pulps</i>	- 114 -

3.4.7.1	<i>Confirming the Expression of Angiogenic Growth Factor mRNA in Human Dental Pulps and MG-63 Osteosarcoma Cells.....</i>	<i>- 114 -</i>
3.4.7.2	<i>Determining the Relative Expression of Angiogenic Growth Factor mRNA in Human Dental Pulps.....</i>	<i>- 115 -</i>
3.4.7.2.a	<i>Comparing mRNA Expression using the Livak Comparative Ct Method.....</i>	<i>- 115 -</i>
3.4.7.2.b	<i>Comparing mRNA Expression using Pfaffl's REST Method.....</i>	<i>- 120 -</i>
3.5	Discussion	- 125 -
3.5.1	Sample Storage and RNA Preservation	- 125 -
3.5.2	RNA Isolation, Yield and Sample Purity	- 126 -
3.5.2.1	RNA Isolation Protocols.....	- 126 -
3.5.2.2	Assessment of RNA Integrity.....	- 129 -
3.5.2.3	Optimisation of Protocols for Quantification of RNA Yields.....	- 130 -
3.5.3	Optimisation of Protocols for RT-PCR and RT-qPCR	- 131 -
3.5.3.1	Primer Design.....	- 131 -
3.5.3.2	Standardising Amount of RNA Input and Reaction Reagents	- 132 -
3.5.3.3	One-Step RT-qPCR vs RNA Amplification : Getting the most out of samples with small RNA _{total} yields	- 134 -
3.5.4	Optimising Protocols for Normalising RT-qPCR Data	- 135 -
3.5.4.1	Normalising Against RNA Input Amount	- 136 -
3.5.4.2	Assessing the Variance in Reference Gene Expression	- 136 -
3.5.4.3	Identifying a Stable Reference Gene: BestKeeper Analysis.....	- 137 -
3.5.4.4	Identifying a Stable Reference Gene: geNorm and NormFinder Analysis	- 138 -
3.5.4.5	Reference Genes: The More the Merrier.....	- 139 -
3.5.5	Relative Expression Analysis of RT-qPCR Data from Orthodontically Treated Pulps.....	- 140 -
3.5.5.1	Delta C _t and Delta-Delta C _t	- 140 -

3.5.5.2	<i>Correcting for Reaction Efficiency using the Relative Expression Software Tool.....</i>	<i>- 142 -</i>
3.5.5.3	<i>Normalising RT-qPCR Data against a Single Reference Gene Compared to a Pair of Reference Genes.....</i>	<i>- 143 -</i>
3.5.5.4	<i>Normalising RT-qPCR Data against Reference Genes Identified as Unstable under our Experimental Conditions.....</i>	<i>- 144 -</i>
3.6	Conclusion	- 145 -

Chapter IV Effects of Orthodontic Force Application on mRNA and Protein Expression of Angiogenic Growth

	Factors in Human Dental Pulp	- 147 -
4.1	Introduction.....	- 148 -
4.2	Aims	- 151 -
4.3	Materials and Methods.....	- 152 -
	MATERIALS.....	- 152 -
4.3.1	<i>Human Dental Pulps</i>	<i>- 152 -</i>
	METHODS	- 152 -
4.3.2	<i>Orthodontic Force Application and Dental Pulp Isolation</i>	<i>- 152 -</i>
4.3.3	<i>RNA Isolation.....</i>	<i>- 154 -</i>
4.3.4	<i>RNA Quantification.....</i>	<i>- 154 -</i>
4.3.5	<i>Reverse-Transcription.....</i>	<i>- 155 -</i>
4.3.6	<i>Selection of Angiogenesis-Specific Genes for mRNA Expression Analysis</i>	<i>- 155 -</i>
4.3.7	<i>RT-qPCR Analysis of mRNA Expression</i>	<i>- 157 -</i>
4.3.8	<i>Statistical Analysis of RT-qPCR Data.....</i>	<i>- 166 -</i>

4.3.9	<i>Pulp Tissue Homogenisation for Protein Analysis</i>	- 167 -
4.3.10	<i>Quantification of Proteins Associated with Angiogenesis</i>	- 168 -
4.4	Results	- 169 -
4.4.1	<i>Relative mRNA Expression Analysis of RT-qPCR Data</i>	- 169 -
4.4.2	<i>Assessment of Biological Variability in RT-qPCR Data.....</i>	- 173 -
4.4.3	<i>Linking Trends in Biological Replicates to those in RT-qPCR Data.....</i>	- 180 -
4.4.3.1	<i>The Effect of Phenotypic Factors on mRNA Expression Levels</i>	- 183 -
4.4.3.2	<i>The Effect of Orthodontic Force Duration on mRNA Expression Levels.....</i>	- 187 -
4.4.3.3	<i>Identifying Trends in the Variability of mRNA Expression across all Treatment Time-Points.....</i>	- 188 -
4.4.4	<i>Statistical Standardisation of Biological Variability in RT-qPCR Data.....</i>	- 189 -
4.4.5	<i>Linking Trends in Protein Expression Profiles to Orthodontic Force Application.....</i>	- 194 -
4.4.6	<i>Linking Trends in Biological Replicates to Protein Expression Profiles</i>	- 196 -
4.4.6.1	<i>Linking Patient Phenotypes to Protein Expression Profiles</i>	- 199 -
4.4.7	<i>Linking mRNA Expression to Protein Expression in Human Dental Pulp.....</i>	- 201 -
4.5	Discussion	- 206 -
4.5.1	<i>Validating the use of Commercially Purchased Primers.....</i>	- 207 -
4.5.2	<i>Relative Expression Analysis Showed Minimal Differences in mRNA Expression at any Treatment Time-Point</i>	- 207 -

4.5.3	<i>Biological Variability May Play a Significant Role in RT-qPCR mRNA Expression Data Analysis.....</i>	208 -
4.5.4	<i>Investigating Changes within Individuals to Determine the Effect of Force Application on mRNA Expression of Angiogenesis-Specific Genes.....</i>	209 -
4.5.5	<i>Linking Biological Variability in Control Samples to Phenotypic Factors</i>	211 -
4.5.6	<i>The Effect of Force Application on mRNA Expression: A Regression Analysis</i>	214 -
4.5.7	<i>Is the mRNA Expression of Angiogenesis-Specific Genes in the Human Dental Pulp Linked?.....</i>	220 -
4.5.8	<i>Correcting for Biological Variability Allows for Statistically Valid Conclusions to be Drawn.....</i>	222 -
4.5.9	<i>Determining Changes in Protein Expression by ELISA</i>	228 -
4.5.10	<i>Investigating Changes within Individuals to Determine the Effect of Force Application on Protein Expression at Distinct Treatment Time-Points.....</i>	236 -
4.5.11	<i>Linking Protein Expression to Phenotypic Factors</i>	239 -
4.5.12	<i>Linking mRNA Expression to Protein Expression</i>	240 -
4.6	<i>Conclusion</i>	243 -

Chapter V Effects of Hypoxia on the Angiogenic Response

	in Human Dental Pulp Explants.....	249 -
5.1	Introduction.....	250 -
5.2	Aims	253 -

5.3	Materials and Methods.....	- 254 -
	MATERIALS.....	- 254 -
5.3.1	<i>Human Dental Pulps</i>	- 254 -
	METHODS	- 254 -
5.3.2	<i>Tooth Extraction and Dental Pulp Isolation</i>	- 254 -
5.3.3	<i>Exposure of Human Dental Pulps to Hypoxia</i>	- 255 -
5.3.4	<i>Validation of Hypoxic Exposure</i>	- 259 -
5.3.5	<i>RNA Isolation</i>	- 259 -
5.3.6	<i>RNA Quantification</i>	- 260 -
5.3.7	<i>Reverse-Transcription</i>	- 260 -
5.3.8	<i>RT-qPCR Analysis of mRNA Expression</i>	- 260 -
5.3.9	<i>Statistical Analysis of RT-qPCR Data</i>	- 261 -
5.3.10	<i>Pulp Tissue Homogenisation for Protein Analysis</i>	- 262 -
5.3.11	<i>Quantification of Proteins Associated with Angiogenesis</i>	- 262 -
5.4	Results.....	- 264 -
5.4.1	<i>Verification of Hypoxic Exposure by Measuring Oxygen Tension</i>	- 264 -
5.4.2	<i>Statistical Standardisation of Biological Variability in RT-qPCR</i> <i>Data and Relative Expression Analysis</i>	- 267 -
5.4.3	<i>The Effect of Phenotypic Factors on mRNA Expression Levels</i>	- 272 -
5.4.4	<i>The Effect of Hypoxic Intensity on mRNA Expression Levels</i>	- 274 -
5.4.5	<i>Protein Expression Profiles as a Response to Hypoxia</i>	- 277 -
5.4.6	<i>Linking Patient Phenotypes to Protein Expression Profiles</i>	- 282 -
5.4.7	<i>The Effect of an Extended Recovery Period on Protein Expression</i>	- 282 -
5.5	Discussion	- 286 -

5.5.1	<i>Verification of the Intensity of Hypoxic Exposure Experienced by Human Dental Pulps by Measuring Oxygen Tension</i>	- 287 -
5.5.2	<i>Relative Expression of Angiogenesis-Specific mRNA in Pulps Exposed to Hypoxia</i>	- 289 -
5.5.2.1	<i>Intermittent Hypoxia Group</i>	- 289 -
5.5.2.2	<i>Repeated Intermittent Hypoxia Group</i>	- 294 -
5.5.2.3	<i>Chronic Hypoxia Group</i>	- 299 -
5.5.3	<i>The Effect of Phenotypic Factors and Hypoxic Exposure on mRNA Expression</i>	- 301 -
5.5.4	<i>The Effect of Hypoxic Intensity on mRNA Expression</i>	- 304 -
5.5.5	<i>Protein Expression Profiles as a Response to Hypoxia</i>	- 307 -
5.5.5.1	<i>Intermittent Hypoxia</i>	- 309 -
5.5.5.2	<i>Repeated Intermittent Hypoxia</i>	- 312 -
5.5.5.3	<i>Chronic Hypoxia</i>	- 313 -
5.5.5.4	<i>Inter-treatment Trends of Protein Expression</i>	- 315 -
5.5.6	<i>Linking Patient Phenotypes to Protein Expression Profiles</i>	- 317 -
5.5.7	<i>The Effect of an Extended Recovery Period of Protein Expression</i>	- 319 -
5.5.8	<i>Will Tissue Culture Provide a Representation of the True Physiological Response?</i>	- 320 -
5.6	<i>Conclusion</i>	- 323 -
	Chapter VI General Discussion	- 328 -
6.1.1	<i>Experimental Model for Orthodontic Force Application</i>	- 329 -
6.1.2	<i>Orthodontic Force Intensity</i>	- 330 -
6.1.3	<i>Optimisation of Experimental Protocols for mRNA Expression Analysis</i>	- 330 -

6.1.3.1	<i>Sample Storage and RNA Preservation</i>	- 331 -
6.1.3.2	<i>Sample Homogenisation.....</i>	- 331 -
6.1.3.3	<i>RNA Isolation Protocols.....</i>	- 331 -
6.1.3.4	<i>RNA Quantification Protocols</i>	- 332 -
6.1.3.5	<i>Reverse-Transcription Protocols.....</i>	- 333 -
6.1.3.6	<i>Normalisation of RT-qPCR Data: Reference Gene vs. Normalising Factor.....</i>	- 334 -
6.1.3.7	<i>The Effect of Reaction Efficiency on mRNA Quantification</i>	- 335 -
6.1.3.8	<i>Correcting for Biological Variability in mRNA Expression Data</i>	- 338 -
6.1.4	<i>Angiogenic Response of the Human Dental Pulp to Orthodontic Force Application.....</i>	- 339 -
6.1.5	<i>The Response of the Human Dental Pulp to Varied Intensities of Hypoxic Exposure</i>	- 343 -
6.1.6	<i>The Effect of Patient Phenotypes on mRNA and Protein Abundances.....</i>	- 348 -
6.1.7	<i>Linking Responses of Orthodontically Treated Pulp to the Characterised Responses of Pulp Exposed to Varied Hypoxic Intensities</i>	- 349 -
Chapter VII	Conclusions and Future Work	- 354 -
7.1	Conclusions.....	- 355 -
7.2	Future Work.....	- 361 -
Chapter VIII	Appendix	- 367 -
References	- 369 -

Declaration of Word Count

I, Timarah Grace Willson, declare that the word count for the text within this thesis does not exceed the upper limit of 100 000 words. The word count per chapter is as follows:

	Abstract	300
	Aims of the Thesis	140
Chapter 1	Introduction	6,758
Chapter 2	Materials and Methods	1,106
Chapter 3	Optimising Protocols for the Analysis of Human Dental Pulp at the Molecular Level	15,975
Chapter 4	Effects of Orthodontic Force Application on mRNA and Protein Expression of Angiogenic Growth Factors in Human Dental Pulp	22,021
Chapter 5	Effects of Hypoxia of the Angiogenic Response in Human Dental Pulp Explants	18,663
Chapter 6	General Discussion	7,677
Chapter 7	Conclusions and Future Work	3,403
Chapter 8	Appendix – Comment from Author	156
TOTAL WORD COUNT:		76,199

List of Presentations

- **MPhil to PhD Transfer Presentation**, Floor 22 Guy's Wing, Guy's Campus, King's College London, London, UK, Monday 10th September 2007.
'Angiogenic Growth Factors in Human Dental Pulp.'
- **Dental Institute Annual Postgraduate Day**, Floor 18 Guy's Wing, Guy's Campus, King's College London, London, UK, Wednesday 16th April 2008.
'The expression of angiogenic growth factors in the human dental pulp following orthodontic force application.'
- **PEF/IADR Conference**, Poster Hall 2, Queen Elizabeth II Conference Centre, London, UK, Wednesday 10th September 2008. *'Angiogenic Growth Factors in human dental pulp.'* Abstract 238 (Willson *et al.* 2008)
- **Dental Institute Annual Postgraduate Day**, Floor 18 Guy's Wing, Guy's Campus, King's College London, London, UK, Wednesday 25th March 2009.
'The expression of angiogenic growth factors in the human dental pulp following orthodontic force application.'

Certificate and prize awarded by Prof Nairn Wilson, Dean and Head of the Dental Institute, for the best presentation given within the session of the postgraduate research day.

- **IADR General Session**, Exhibit Hall D, Miami Beach Convention Center, Florida, U.S.A, Friday 3rd April 2009. '*Analysis of RNA from Human Dental Pulp samples.*' Abstract 1781 (Willson *et al.* 2009).
- **London Oral Biology Club**, Garrod Building, Barts and the London School of Medicine and Dentistry, London, UK, Wednesday 22nd April 2009. '*Analysis of RNA from Human Dental Pulp samples.*'

List of Abbreviations

Units of Measurement

%	percent
°C	degrees Celsius/Centigrade
µg	microgram
µg/µl	microgram per microlitre
µg/ml	microgram per millilitre
µl	microlitre
µm	micrometre
µmol	micromole
µM	micromolar
bp	base pair
cm ²	centimetre squared
g	centrifugal G-force (when applied to acceleration)
g	gram (when applied to a mass)
grams-force	1 gram x 9.80665 m ^{-sec²}
G	gauge (not the gravitational constant)
hr	hour
kb	kilo base
kDa	kilo Dalton
M	molar (moles per litre)
min	minute
ml	millilitre

mM	millimolar
mmol	millimole
ms ⁻¹	metres per second
N	Newton
n	number of samples
ng	nanogram
ng/reaction	nanogram per reaction
ng/μl	nanogram per microlitre
nm	nanometre
p	probability
pg/ml	picogram per millilitre
pg/μl	picogram per microlitre
pH	acidity/alkalinity scale
pM/μl	picomolar per microlitre
pmol/μl	picomole per microlitre
ppm	parts per million
psi	pounds per square inch
sec	second
U/ml	units per volume
V	Volt

Other Abbreviations

A_{230}	Absorbance of light at a wavelength of 230 nm which corresponds with organic compound contamination
$A_{260}:A_{230}$	Ratio of absorbance of light at wavelengths 260 nm to 230 nm (for nucleic acids and organic compound contamination, respectively)
A_{260}	Absorbance of light at 260 nm which corresponds with nucleic acids
$A_{260}:A_{280}$	Ratio of absorbance of light at wavelengths 260 nm to 280 nm (for nucleic acids and organic proteins, respectively)
A_{280}	Absorbance of light at 280 nm which corresponds with organic proteins
ACTB	beta actin
ANGPT1	angiopoietin 1
ANOVA	Analysis of Variance
<i>a priori</i>	before
ATP	Adenosine triphosphate
BCA	bicinchoninic acid
BLAST	Basic Local Alignment Search Tool
Cat. No.	catalogue number
CD34 ⁺⁺	cluster of differentiation 34
CD51	Integrin alpha V
CD61	Integrin beta 3
CD105 ⁺⁺	Endolgin

CD146 ⁺⁺	melanoma cell adhesion molecule (MCAM)
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
CO ₂	carbon dioxide
C _q	quantification cycle
C _t	cycle threshold
CV	Coefficient of Variation
CXCL8	chemokine (C-X-C motif) ligand 8
Delta C _t	change in cycle threshold between gene of interest and normalising factor
Delta-Delta C _t	change in cycle threshold between control and treated samples
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphates
E	efficiency of the slope
E _{GOI}	efficiency of the gene of interest
E _{REF}	efficiency of the reference gene/normalising factor
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
<i>et al.</i>	and others
FACS	fluorescence-activated cell sorting
FACTT	Fluorescent Amplification Catalysed by T7 polymerase Technique

FCS	fetal calf serum
FGF2	fibroblast growth factor 2 (basic)
FOXY probe	fibre optic oxygen probe
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC content	guanine-cytosine content
GOI	gene of interest
GOIs	genes of interest
GTPase	guanosine triphosphates hydrolase enzyme
HIF1	hypoxia inducible factor 1
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
HIV	human immunodeficiency virus
HPRT1	hypoxanthine phosphoribosyltransferase 1
Hsp90	heat shock protein 90
HUVEC	human umbilical vein endothelial cell
IL8	interleukin 8
<i>in silico</i>	in simulation
<i>in situ</i>	in place
<i>in vitro</i>	in glass
<i>in vivo</i>	in life
ITGAV	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
ITGB8	integrin, beta 8
KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)

KEGG	Kyoto Encyclopaedia of Genes and Genomes
LOD	limit of detection
MAPK	mitogen-activated protein kinase
MEM	minimum essential medium
Mg ²⁺	magnesium
MG-63	human osteosarcoma cell line
MgCl ²⁺	magnesium chloride
MIQE	<i>Minimum Information for publication of Quantitative real-time PCR Experiments</i>
miRNA	micro ribonucleic acid
MMLV	Moloney Murine Leukemia Virus
MMLV-RT	Moloney Murine Leukemia Virus reverse transcriptase
MMP2	matrix metalloproteinase 2 (gelatinase A, 72kDa type IV collagenase)
mRNA	messenger ribonucleic acid
mRNA:rRNA	Ratio of messenger ribonucleic acid to ribosomal ribonucleic acid
M-value	gene expression stability value from GeNorm analysis
N ₂	nitrogen
NEDD4L	neural precursor cell expressed, developmentally gene
NGF	nerve growth factor
NOS2	nitric oxide synthase 2, inducible
NTC	no template control
O ₂	oxygen
ODD	oxygen-dependent degradation domain
Oligo-DT	deoxy-thymidine nucleotides

PARE	Protein Abundance and mRNA Expression data tool
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFA	platelet-derived growth factor alpha polypeptide
PDGF-AA	platelet-derived growth factor alpha polypeptide homodimer
PDGF-AB	platelet-derived growth factor alpha-beta polypeptide heterodimer
PDGFB	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)
PDGF-BB	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog) homodimer
PDGFRA	platelet-derived growth factor alpha receptor polypeptide
PDGFRB	platelet-derived growth factor beta receptor polypeptide (simian sarcoma viral (v-sis) oncogene homolog)
PDL	periodontal ligament
Pen-Strep	Penicillin-Streptomycin
PHD	prolylhydroxylase
PI3K-Akt	phosphatidylinositol-3-kinase – protein kinase B
PPIA	peptidylprolyl isomerase A (cyclophilin A)
Primer-BLAST	Primer Basic Local Alignment Search Tool
pVHL	von Hippel-Lindau tumour suppressor protein
qPCR	quantitative real-time polymerase chain reaction
r	coefficient of correlation
r^2	coefficient of determination
Rap1	DNA-binding protein Rap1
Ras	DNA-binding protein Ras

REST-2009	Relative Expression Software Tool-2009
RIN	RNA integrity number
RNA	ribonucleic acid
RNAse	ribonuclease
RNA _{total}	total ribonucleic acid
RQI	RNA quality indicator
ROBO4	roundabout homolog 4, magic roundabout (Drosophila)
RPL13A	ribosomal protein L13A
rRNA	ribosomal ribonucleic acid
RT	reverse transcription
RT control	reverse transcription control
RT-PCR	reverse transcription polymerase chain reaction (legacy)
RT-qPCR	reverse-transcription quantitative real-time polymerase chain reaction
SD	standard deviation
SE	standard error
SELE	selectin E
SEM	standard error of the mean
siRNA	small interfering ribonucleic acid
SMAD7	SMAD family member 7
SMURFs	SMURF E3 ubiquitin-protein ligase
STDEV	standard deviation
TBE	TrisBorateEDTA
TEK	TEK tyrosine kinase, endothelial
TGFB1	transforming growth factor, beta 1

TGFBR1	transforming growth factor, beta receptor 1
tRNA	transfer ribonucleic acid
UBC	ubiquitin C
UCHL5	ubiquitin C-terminal hydrolase L5
USP15	ubiquitin specific peptidase 15
UV	ultraviolet
VEGFA	vascular endothelial growth factor alpha
vSMC	vascular smooth muscle cells
YWHAZ	tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein, zeta polypeptide

List of Figures

FIGURE 1	STAGES OF TOOTH DEVELOPMENT	- 46 -
FIGURE 2	BIOANALYSIS OF RNA FROM PULP HOMOGENISED BY MORTAR AND PESTLE.....	- 100 -
FIGURE 3	BIOANALYSIS OF RNA FROM PULP HOMOGENISED BY PASSING PULP THROUGH A NEEDLE.....	- 101 -
FIGURE 4	BIOANALYSIS OF RNA FROM PULP HOMOGENISED BY FASTPREP®-24	- 101 -
FIGURE 5	BIOANALYSIS OF RNA FROM PULP HOMOGENISED IN A GLASS DOUNCE TISSUE HOMOGENISER	- 102 -
FIGURE 6	OPTIMISATION OF REVERSE-TRANSCRIPTION PROTOCOLS FOR RT-PCR.....	- 105 -
FIGURE 7	DISTRIBUTION OF CANDIDATE REFERENCE GENE EXPRESSION IN CONTROL AND TREATED SAMPLES.....	- 109 -
FIGURE 8	ANALYSIS OF EXPRESSION STABILITY OF CANDIDATE REFERENCE GENES USING THE GENORM ALGORITHM	- 112 -
FIGURE 9	ANALYSIS OF EXPRESSION STABILITY OF CANDIDATE REFERENCE GENES USING THE NORMFINDER ALGORITHM: INTRA-GROUP VARIATION	- 114 -
FIGURE 10	GEL ELECTROPHORETOGRAMS OF RT-PCR PRODUCTS IN THE MG-63 OSTEOSARCOMA CELL LINE AND AN UNTREATED HUMAN DENTAL PULP.....	- 115 -
FIGURE 11	DIFFERENTIAL EXPRESSION ANALYSIS OF ANGIOGENIC GROWTH FACTOR MRNA IN TWO WEEK TREATED SAMPLES RELATIVE TO CONTRALATERAL CONTROLS. NORMALISING AGAINST UBC.....	- 121 -
FIGURE 12	DIFFERENTIAL EXPRESSION ANALYSIS OF ANGIOGENIC GROWTH FACTOR MRNA IN TWO WEEK TREATED SAMPLES RELATIVE TO CONTRALATERAL CONTROLS. NORMALISING AGAINST THE GEOMETRIC MEAN OF RPL13A AND UBC.....	- 122 -
FIGURE 13	NORMALISATION OF DIFFERENTIAL EXPRESSION ANALYSIS DATA WITH MULTIPLE REFERENCE GENES	- 123 -
FIGURE 14	DIFFERENTIAL EXPRESSION ANALYSIS OF ANGIOGENIC GROWTH FACTOR MRNA IN ONE DAY TREATED SAMPLES RELATIVE TO CONTRALATERAL CONTROL. NORMALISING AGAINST THE GEOMETRIC MEAN OF RPL13A AND UBC	- 170 -

FIGURE 15	DIFFERENTIAL EXPRESSION ANALYSIS OF ANGIOGENIC GROWTH FACTOR MRNA EXPRESSION IN THREE DAY TREATED SAMPLES RELATIVE TO CONTRALATERAL CONTROLS. NORMALISING AGAINST THE GEOMETRIC MEAN OF RPL13A AND UBC .. - 171 -
FIGURE 16	DIFFERENTIAL EXPRESSION ANALYSIS OF ANGIOGENIC GROWTH FACTOR MRNA EXPRESSION IN FOURTEEN DAY TREATED SAMPLES RELATIVE TO CONTRALATERAL CONTROLS. NORMALISING AGAINST THE GEOMETRIC MEAN OF RPL13A AND UBC .. - 172 -
FIGURE 17	FOLD-CHANGE EXPRESSION ANALYSIS OF ANGIOGENIC GROWTH FACTOR MRNA IN ONE DAY TREATED SAMPLES RELATIVE TO CONTRALATERAL CONTROLS. NORMALISING AGAINST THE GEOMETRIC MEAN OF RPL13A AND UBC..... - 191 -
FIGURE 18	FOLD-CHANGE EXPRESSION ANALYSIS OF ANGIOGENIC GROWTH FACTOR MRNA IN THREE DAY TREATED SAMPLES RELATIVE TO CONTRALATERAL CONTROLS. NORMALISING AGAINST THE GEOMETRIC MEAN OF RPL13A AND UBC..... - 192 -
FIGURE 19	FOLD-CHANGE EXPRESSION ANALYSIS OF ANGIOGENIC GROWTH FACTOR MRNA IN TWO WEEK (14 DAY) TREATED SAMPLES RELATIVE TO CONTRALATERAL CONTROLS. NORMALISING AGAINST THE GEOMETRIC MEAN OF RPL13A AND UBC..... - 193 -
FIGURE 20	INDUCING CHRONIC AND INTERMITTENT HYPOXIA IN THE HUMAN DENTAL PULP - 257 -
FIGURE 21	COMPARISON OF OXYGEN PROBES FOR VALIDATION OF HYPOXIA EXPOSURE..... - 266 -
FIGURE 22	FOLD CHANGE EXPRESSION ANALYSIS OF ANGIOGENIC GROWTH FACTOR MRNA IN PULP EXPLANTS EXPOSED TO INTERMITTENT HYPOXIA RELATIVE TO NORMOXIC CONTROLS. NORMALISING AGAINST THE GEOMETRIC MEAN OF RPL13A AND UBC .. - 269 -
FIGURE 23	FOLD CHANGE EXPRESSION ANALYSIS OF ANGIOGENIC GROWTH FACTOR MRNA IN PULP EXPLANTS EXPOSED REPEATEDLY TO CYCLES OF INTERMITTENT HYPOXIA RELATIVE TO NORMOXIC CONTROLS. NORMALISING AGAINST THE GEOMETRIC MEAN OF RPL13A AND UBC - 270 -
FIGURE 24	FOLD CHANGE EXPRESSION ANALYSIS OF ANGIOGENIC GROWTH FACTOR MRNA IN PULP EXPLANTS EXPOSED TO CHRONIC HYPOXIA RELATIVE TO NORMOXIC CONTROLS. NORMALISING AGAINST THE GEOMETRIC MEAN OF RPL13A AND UBC..... - 271 -

List of Tables

TABLE 1	CANDIDATE REFERENCE GENES	- 84 -
TABLE 2	CANDIDATE ANGIOGENIC GROWTH FACTORS	- 85 -
TABLE 3	PRIMER SEQUENCES FOR ANGIOGENIC GROWTH FACTOR FGF2 FOR RT-PCR	- 86 -
TABLE 4	PRIMER SEQUENCES FOR ANGIOGENIC GROWTH FACTOR PDGFA FOR RT-PCR	- 86 -
TABLE 5	PRIMER SEQUENCES FOR ANGIOGENIC GROWTH FACTOR TGFB1 FOR RT-PCR	- 87 -
TABLE 6	PRIMER SEQUENCES FOR ANGIOGENIC GROWTH FACTOR VEGFA FOR RT-PCR	- 87 -
TABLE 7	PRIMER SEQUENCES FOR REFERENCE GENE GAPDH FOR RT-PCR	- 88 -
TABLE 8	PRIMER SEQUENCES FOR ANGIOGENIC GROWTH FACTOR FGF2 FOR RT-QPCR	- 88 -
TABLE 9	PRIMER SEQUENCES FOR ANGIOGENIC GROWTH FACTOR PDGFA FOR RT-QPCR	- 89 -
TABLE 10	PRIMER SEQUENCES FOR ANGIOGENIC GROWTH FACTOR TGFB1 FOR RT-QPCR	- 89 -
TABLE 11	PRIMER SEQUENCES FOR ANGIOGENIC GROWTH FACTOR VEGFA FOR RT-QPCR	- 90 -
TABLE 12	PRIMER SEQUENCES FOR CANDIDATE REFERENCE GENE ACTB FOR RT-QPCR	- 90 -
TABLE 13	PRIMER SEQUENCES FOR CANDIDATE REFERENCE GENE GAPDH FOR RT-QPCR	- 91 -
TABLE 14	PRIMER SEQUENCES FOR CANDIDATE REFERENCE GENE HPRT1 FOR RT-QPCR	- 91 -
TABLE 15	PRIMER SEQUENCES FOR CANDIDATE REFERENCE GENE PPIA FOR RT-QPCR	- 92 -
TABLE 16	PRIMER SEQUENCES FOR CANDIDATE REFERENCE GENE RPL13A FOR RT-QPCR	- 92 -
TABLE 17	PRIMER SEQUENCES FOR CANDIDATE REFERENCE GENE UBC FOR RT-QPCR	- 93 -
TABLE 18	PRIMER SEQUENCES FOR CANDIDATE REFERENCE GENE YWHAZ FOR RT-QPCR	- 93 -
TABLE 19	BIOANALYZER RIN VALUES AND GEL ELECTROPHORETOGRAMS	- 99 -
TABLE 20	RNA _{TOTAL} YIELDS FROM HUMAN DENTAL PULPS WHEN QUANTIFIED BY NANODROP SPECTROPHOTOMETRY AND FLUORESCENCE (RIBOGREEN ASSAY)	- 103 -
TABLE 21	COMPARISON BETWEEN THE ISCRIPIT ONE-STEP RT-QPCR KIT AND THE WT-OVATION ™ PICO RNA AMPLIFICATION SYSTEM	- 106 -
TABLE 22	NORMALISATION OF CANDIDATE REFERENCE GENE EXPRESSION AGAINST TOTAL RNA	- 108 -
TABLE 23	ANALYSIS OF VARIANCE AND STABILITY OF EACH CANDIDATE REFERENCE GENE USING THE <i>BESTKEEPER</i> ® TOOL	- 110 -
TABLE 24	ANALYSIS OF INTER-REFERENCE GENE RELATIONS USING PAIR-WISE CORRELATION AND REGRESSION ANALYSIS IN THE <i>BESTKEEPER</i> © EXCEL-BASED TOOL	- 111 -

TABLE 25	ANALYSIS OF EXPRESSION STABILITY OF CANDIDATE REFERENCE GENES USING THE NORMFINDER ALGORITHM – WITHOUT GROUPS	113 -
TABLE 26	DELTA C_T AND DELTA-DELTA C_T ANALYSIS OF ANGIOGENIC GROWTH FACTOR FGF2 NORMALISED AGAINST THE REFERENCE GENE UBC	116 -
TABLE 27	DELTA C_T AND DELTA-DELTA C_T ANALYSIS OF ANGIOGENIC GROWTH FACTOR PDGFA NORMALISED AGAINST THE REFERENCE GENE UBC.....	116 -
TABLE 28	DELTA C_T AND DELTA-DELTA C_T ANALYSIS OF ANGIOGENIC GROWTH FACTOR TGFB1 NORMALISED AGAINST THE REFERENCE GENE UBC	117 -
TABLE 29	DELTA C_T AND DELTA-DELTA C_T ANALYSIS OF ANGIOGENIC GROWTH FACTOR VEGFA NORMALISED AGAINST THE REFERENCE GENE UBC	117 -
TABLE 30	DELTA C_T AND DELTA-DELTA C_T ANALYSIS OF ANGIOGENIC GROWTH FACTOR FGF2 NORMALISED AGAINST THE GEOMETRIC MEAN EXPRESSION OF THE REFERENCE GENES RPL13A AND UBC	118 -
TABLE 31	DELTA C_T AND DELTA-DELTA C_T ANALYSIS OF ANGIOGENIC GROWTH FACTOR PDGFA NORMALISED AGAINST THE GEOMETRIC MEAN EXPRESSION OF THE REFERENCE GENES RPL13A AND UBC	118 -
TABLE 32	DELTA C_T AND DELTA-DELTA C_T ANALYSIS OF ANGIOGENIC GROWTH FACTOR TGFB1 NORMALISED AGAINST THE GEOMETRIC MEAN EXPRESSION OF THE REFERENCE GENES RPL13A AND UBC	119 -
TABLE 33	DELTA C_T AND DELTA-DELTA C_T ANALYSIS OF ANGIOGENIC GROWTH FACTOR VEGFA NORMALISED AGAINST THE GEOMETRIC MEAN EXPRESSION OF THE REFERENCE GENES RPL13A AND UBC	119 -
TABLE 34	EXPANDED LIST OF ANGIOGENESIS-SPECIFIC GENES FOR MRNA EXPRESSION ANALYSIS	156 -
TABLE 35	PRIMERDESIGN LTD. UK PRIMER SEQUENCES OF THE ANGIOGENESIS-SPECIFIC GENE ANGPT1 FOR RT-QPCR.....	158 -
TABLE 36	PRIMERDESIGN LTD. UK PRIMER SEQUENCES OF THE ANGIOGENESIS-SPECIFIC GENE FGF2 FOR RT-QPCR.....	158 -
TABLE 37	PRIMERDESIGN LTD. UK PRIMER SEQUENCES OF THE ANGIOGENESIS-SPECIFIC GENE HIF1A FOR RT-QPCR.....	159 -

TABLE 38	PRIMERDESIGN LTD. UK PRIMER SEQUENCES OF THE ANGIOGENESIS-SPECIFIC GENE IL8 FOR RT-QPCR	- 159 -
TABLE 39	PRIMERDESIGN LTD. UK PRIMER SEQUENCES OF THE ANGIOGENESIS-SPECIFIC GENE ITGAV FOR RT-QPCR.....	- 160 -
TABLE 40	PRIMERDESIGN LTD. UK PRIMER SEQUENCES OF THE ANGIOGENESIS-SPECIFIC GENE ITGB3 FOR RT-QPCR.....	- 160 -
TABLE 41	PRIMERDESIGN LTD. UK PRIMER SEQUENCES OF THE ANGIOGENESIS-SPECIFIC GENE KDR FOR RT-QPCR.....	- 161 -
TABLE 42	PRIMERDESIGN LTD. UK PRIMER SEQUENCES OF THE ANGIOGENESIS-SPECIFIC GENE MMP2 FOR RT-QPCR.....	- 161 -
TABLE 43	PRIMERDESIGN LTD. UK PRIMER SEQUENCES OF THE ANGIOGENESIS-SPECIFIC GENE NOS2 FOR RT-QPCR	- 162 -
TABLE 44	PRIMERDESIGN LTD. UK PRIMER SEQUENCES OF THE ANGIOGENESIS-SPECIFIC GENE PDGFA FOR RT-QPCR	- 162 -
TABLE 45	PRIMERDESIGN LTD. UK PRIMER SEQUENCES OF THE ANGIOGENESIS-SPECIFIC GENE PDGFB FOR RT-QPCR	- 163 -
TABLE 46	PRIMERDESIGN LTD. UK PRIMER SEQUENCES OF THE ANGIOGENESIS-SPECIFIC GENE ROBO4 FOR RT-QPCR	- 163 -
TABLE 47	PRIMERDESIGN LTD. UK PRIMER SEQUENCES OF THE ANGIOGENESIS-SPECIFIC GENE SELE FOR RT-QPCR.....	- 164 -
TABLE 48	PRIMERDESIGN LTD. UK PRIMER SEQUENCES OF THE ANGIOGENESIS-SPECIFIC GENE TEK FOR RT-QPCR	- 164 -
TABLE 49	PRIMERDESIGN LTD. UK PRIMER SEQUENCES OF THE ANGIOGENESIS-SPECIFIC GENE TGFB1 FOR RT-QPCR.....	- 165 -
TABLE 50	PRIMERDESIGN LTD. UK PRIMER SEQUENCES OF THE ANGIOGENESIS-SPECIFIC GENE TGFB1 FOR RT-QPCR	- 165 -
TABLE 51	PRIMERDESIGN LTD. UK PRIMER SEQUENCES OF THE ANGIOGENESIS-SPECIFIC GENE VEGFA FOR RT-QPCR.....	- 166 -
TABLE 52	VARIABILITY OF ANGPT1, FGF2 AND HIF1A MRNA EXPRESSION IN CONTROL SAMPLES	- 174 -

TABLE 53	VARIABILITY OF IL8, ITGAV AND ITGB3 mRNA EXPRESSION IN CONTROL SAMPLES	- 175 -
TABLE 54	VARIABILITY OF KDR, MMP2 AND NOS2 mRNA EXPRESSION IN CONTROL SAMPLES	- 176 -
TABLE 55	VARIABILITY OF PDGFA, PDGFB AND ROBO4 mRNA EXPRESSION IN CONTROL SAMPLES	- 177 -
TABLE 56	VARIABILITY OF SELE, TEK AND TGFB1 mRNA EXPRESSION IN CONTROL SAMPLES	- 178 -
TABLE 57	VARIABILITY OF TGFB1 AND VEGFA mRNA EXPRESSION IN CONTROL SAMPLES	- 179 -
TABLE 58	INTER- AND INTRA-PATIENT TRENDS IN mRNA EXPRESSION FOLLOWING 1 DAY ORTHODONTIC FORCE APPLICATION	- 180 -
TABLE 59	INTER- AND INTRA-PATIENT TRENDS IN mRNA EXPRESSION FOLLOWING 3 DAYS OF ORTHODONTIC FORCE APPLICATION	- 181 -
TABLE 60	INTER- AND INTRA-PATIENT TRENDS IN mRNA EXPRESSION FOLLOWING 2 WEEKS (14 DAYS) OF ORTHODONTIC FORCE APPLICATION	- 182 -
TABLE 61	DETAILS OF PATIENTS' AGE, GENDER, ETHNICITY AND ROOT MORPHOLOGY	- 185 -
TABLE 62	MULTIVARIATE MIXED LINEAR REGRESSION ANALYSIS IDENTIFIES THE IMPACT OF PATIENT PHENOTYPE ON mRNA EXPRESSION	- 186 -
TABLE 63	ONE-WAY ANOVA AND PAIR-WISE REGRESSION ANALYSIS OF THE EFFECT OF ORTHODONTIC FORCE APPLICATION AND THE DURATION OF TREATMENT ON mRNA EXPRESSION	- 188 -
TABLE 64	COMMONALITY OF VARIABILITY IN mRNA EXPRESSION OF ANGIOGENESIS-SPECIFIC GOIs	- 189 -
TABLE 65	ANALYTE CONCENTRATIONS (PG/ML) OF ANGIOGENESIS-SPECIFIC GOIs IN HUMAN DENTAL PULP EXPLANTS EXPOSED TO ONE DAY OF ORTHODONTIC FORCE APPLICATION PRIOR TO TOOTH EXTRACTION	- 194 -
TABLE 66	ANALYTE CONCENTRATIONS (PG/ML) OF ANGIOGENESIS-SPECIFIC GOIs IN HUMAN DENTAL PULP EXPLANTS EXPOSED TO THREE DAYS OF ORTHODONTIC FORCE APPLICATION PRIOR TO TOOTH EXTRACTION	- 195 -

TABLE 67	ANALYTE CONCENTRATIONS (PG/ML) OF ANGIOGENESIS-SPECIFIC GOIS IN HUMAN DENTAL PULP EXPLANTS EXPOSED TO TWO WEEKS OF ORTHODONTIC FORCE APPLICATION PRIOR TO TOOTH EXTRACTION	- 196 -
TABLE 68	INTER- AND INTRA-PATIENT TRENDS IN PROTEIN EXPRESSION FOLLOWING 1 DAY OF ORTHODONTIC FORCE APPLICATION	- 197 -
TABLE 69	INTER- AND INTRA-PATIENTS TRENDS IN PROTEIN EXPRESSION FOLLOWING 3 DAYS OF ORTHODONTIC FORCE APPLICATION	- 198 -
TABLE 70	INTER- AND INTRA-PATIENT TRENDS IN PROTEIN EXPRESSION FOLLOWING 2 WEEKS OF ORTHODONTIC FORCE APPLICATION	- 198 -
TABLE 71	DETAILS OF PATIENTS' AGE, GENDER, ETHNICITY AND ROOT MORPHOLOGY IN SAMPLES FOR PROTEIN ANALYSIS	- 199 -
TABLE 72	COMPARISON OF mRNA AND PROTEIN EXPRESSION PROFILES IN PATIENTS TREATED WITH ORTHODONTIC FORCE FOR 1 DAY PRIOR TO TOOTH EXTRACTION	- 203 -
TABLE 73	COMPARISON OF mRNA AND PROTEIN EXPRESSION PROFILES IN PATIENTS TREATED WITH ORTHODONTIC FORCE FOR 3 DAYS PRIOR TO TOOTH EXTRACTION	- 204 -
TABLE 74	COMPARISON OF mRNA AND PROTEIN EXPRESSION PROFILES IN PATIENTS TREATED WITH ORTHODONTIC FORCE FOR 2 WEEKS PRIOR TO TOOTH EXTRACTION	- 205 -
TABLE 75	PROTOCOLS FOR INDUCTION OF HYPOXIA IN THE HUMAN DENTAL PULP	- 258 -
TABLE 76	HYPOXIA PROTOCOLS WITH LONG RECOVERY PERIODS FOR PROTEIN PRODUCTION	- 258 -
TABLE 77	AVERAGE OXYGEN CONCENTRATION (%) IN CULTURE MEDIA FOLLOWING HYPOXIC EXPOSURE	- 267 -
TABLE 78	DETAILS OF HYPOXIA PATIENTS' ROOT MORPHOLOGY, ETHNICITY AND GENDER	- 273 -
TABLE 79	UNIVARIATE LINEAR REGRESSION ANALYSIS OF PATIENT PHENOTYPES IN HYPOXICALLY TREATED HUMAN DENTAL PULP EXPLANTS	- 274 -
TABLE 80	ONE-WAY ANOVA AND PAIR-WISE REGRESSION ANALYSIS OF THE EFFECT OF HYPOXIC EXPOSURE AND INTENSITY ON mRNA EXPRESSION LEVELS	- 276 -
TABLE 81	ANALYTE CONCENTRATIONS (PG/ML) OF ANGIOGENESIS-SPECIFIC GOIS IN HUMAN DENTAL PULP EXPLANTS EXPOSED TO INTERMITTENT HYPOXIC CONDITIONS	- 279 -
TABLE 82	ANALYTE CONCENTRATIONS (PG/ML) OF ANGIOGENESIS-SPECIFIC GOIS IN HUMAN DENTAL PULP EXPLANTS EXPOSED TO REPEATED INTERMITTENT HYPOXIC CONDITIONS-	- 280 -

TABLE 83	ANALYTE CONCENTRATIONS (PG/ML) OF ANGIOGENESIS-SPECIFIC GOIS IN HUMAN DENTAL PULP EXPLANTS EXPOSED TO CHRONIC HYPOXIC CONDITIONS - 281 -
TABLE 84	INTER- AND INTRA-PATIENT TRENDS IN PROTEIN EXPRESSION FOLLOWING EXPOSURE TO CHRONIC HYPOXIA AND 16 HR RECOVERY IN SUPPLEMENTED AMBIENT OXYGEN .. - 284 -
TABLE 85	PROTEIN TRENDS FOLLOWING EXPOSURE TO CHRONIC HYPOXIA: A COMPARISON OF SNAP-FREEZING POST EXPOSURE AND PROLONGED INCUBATION - 285 -

Aims of the Thesis

The aims of this study were to:

1. Determine appropriate protocols for sample storage, homogenisation, RNA_{total} isolation, RNA_{total} quantification, reverse-transcription, gene expression normalisation and data analysis in the human dental pulp; thus maximising a single tissue's experimental potential in transcriptomic expression analyses.
2. Determine the transcriptomic and proteomic expression of angiogenesis-specific genes in order to elucidate the angiogenic response of the human dental pulp to the early stages of orthodontic force application.
3. Characterise the angiogenic response of the human dental pulp to defined intensities of hypoxia in order to more fully comprehend the survival mechanisms of this unique tissue.
4. Contrast the angiogenic response of the human dental pulp to orthodontic force application with the angiogenic response of this tissue to various intensities of hypoxia in order to determine whether hypoxia plays a role in routine clinical orthodontic treatment.

Chapter I

Introduction

Introduction

While stem cell isolation and public health issues, such as caries, have brought the human dental pulp to the forefront of translational research (Farges *et al.* 2015, La Noce *et al.* 2014, Werle *et al.* 2016, Winderlich *et al.* 2016, Yusa *et al.* 2016, Zhang *et al.* 2015,); little is currently understood regarding the effects of routine clinical procedures on the behaviour of this specialised tissue. Specifically, the current comprehension of the angiogenic response of the human dental pulp to orthodontic force application is limited, as few studies have been carried out in order to identify and determine key signalling processes in the tissue's response to treatment.

Orthodontic force application has been shown to induce a number of changes in the periodontium, including localised inflammation (Boyd *et al.* 1989, Eliasson *et al.* 1982, Yamaguchi and Kasai 2005), ischemia (Alfuriji *et al.* 2014, Khorsand *et al.* 2013, Rygh 1974, Yamaguchi and Kasai 2005) and reduced blood flow rates in the tissue (d'Apuzzo *et al.* 2013, Patil *et al.* 2013, Zainal *et al.* 2011). It has been hypothesised that the alteration to periodontal blood flow could have an adverse effect on the tightly enclosed dental pulp. A number of studies have investigated changes to the pulpal blood flow rates at the onset of orthodontic treatment and have identified a decrease in flow rate as a direct response (Sano *et al.* 2002, Yamaguchi 2007). Reactive hyperaemia has also been shown to take place in the pulp within half an hour of forces being applied (McDonald and Pitt Ford 1994).

Whether the changes to the pulpal blood flow rates are produced solely as a knock-on effect to the alterations in the periodontium or whether the pulp initiates its own survival mechanism remains unknown. However, several growth factors known to be associated with the angiogenic signalling cascade have been shown to be secreted from the pulp tissue in response to orthodontic treatment (Derringer *et al.* 1996,

Derringer and Linden 1998, Derringer and Linden 2003, Derringer and Linden 2004, Derringer and Linden 2007), indicating that the induced response is a reaction to a negative characteristic of routine force application.

As the tooth undergoes repeated cycles of force loading during the course of orthodontic treatment, a progressive assault on this highly sensitive tissue may be occurring. Therefore it is fundamental that we further investigate the angiogenic response of the human dental pulp in order to: (1) elucidate the degree of assault, (2) identify the cause of the response and (3) assess whether clinical practices require adjustment in order to maintain the integrity of this tissue.

1.1.1 Origins of the Dental Pulp – An Overview of Tooth Development

The tooth organ is known to develop through a series of interactions between the ectodermal epithelium and the underlying neural-crest derived mesenchyme of the first branchial arch during early embryogenesis (Chai *et al.* 2000, Haworth *et al.* 2004, Lumsden 1988, Mina and Kollar 1987, Peters and Balling 1999). These interactions are tightly controlled through the expression of growth factors, transcription factors and morphogens alongside their respective receptors; and have been characterised in the murine model through a number of *in vivo* and *in vitro* experiments.

Following the processes of mapping out the location of future teeth, and determining the number and type of teeth to develop along the mandibular and maxillary arches; the initiation of tooth development is marked by a thickening of the oral epithelium, hereon referred to as the dental lamina, and the formation of an ectodermal placode, which acts as the primary signalling centre in tooth organogenesis (Aberg *et al.* 2004, Cobourne *et al.* 2001, Hardcastle *et al.* 1998, Haworth *et al.* 2004, Liu *et al.* 2008, Mustonen *et al.* 2004, Peters and Balling 1999, Pispa and Thesleff 2003, Salmivirta *et al.* 1996, Thesleff *et al.* 2001).

Reciprocal signalling between the epithelium and the mesenchyme is induced by the ectodermal placode, initiating the process of epithelial cell invagination of the underlying mesenchyme and tooth bud formation, accompanied by concurrent condensation of epithelial and mesenchymal cells at the tip of the bud structure (Cobourne *et al.* 2001, Hardcastle *et al.* 1998, Liu *et al.* 2008, Lumsden 1988, Pispa and Thesleff 2003, Salmivirta *et al.* 1996). Although it is considered that the tooth bud forms as a result of epithelial cell proliferation, the cellular mechanisms governing this process have yet to be fully characterised (Mustonen *et al.* 2004).

The developing tooth now moves from a phase of initiation to a phase of morphogenesis and the signalling for subsequent odontogenesis shifts from an epithelial to a mesenchymal origin (Liu *et al.* 2008, Vaahtokari *et al.* 1996). This process is marked by the formation of the enamel knot, the second signalling centre of tooth development and site for induction of epithelial folding, from the condensed epithelium at the tip of the bud structure during the late-bud stage (Aberg *et al.* 2004, Pispa and Thesleff 2003, Thesleff *et al.* 2001).

Once the enamel knot has fully differentiated, the tooth germ has entered the cap stage of development. At this point, proximal proliferation of the epithelium in conjunction with proliferation of the underlying condensed mesenchyme is induced in order to form the structures associated with this point of morphogenesis, i.e., the dental follicle/sac, the enamel epithelium and the dental papilla (Gritli-Linde *et al.* 2002, Hardcastle *et al.* 1998, Iacob and Veis 2006, Koyama *et al.* 2001, Lesot *et al.* 1996, Matalova *et al.* 2005, Tucker and Sharpe 1999). It has been shown that the size and signal activity of the enamel knot plays a significant role in the size of the crown and number of cusps which will form subsequently (Salazar-Ciudad and Jernvall 2004, Tummers and Thesleff 2009).

As the tooth germ moves through the cap stage toward the bell stage, the epithelium which proliferates in a proximal direction to the enamel knot is folded convexly, enveloping the forming dental papilla. At the tip of each of these folds lies a cervical loop, marking the junction at which the inner and outer enamel epithelium meets (Aberg *et al.* 2004, Jernvall *et al.* 1994, Pispá and Thesleff 2003, Tucker and Sharpe 2004, Vaahtokari *et al.* 1996).

At the late-cap to early-bell stage, the primary enamel knot is removed from the tooth germ by a process of apoptosis. In the incisor, some of the enamel knot cells are maintained in order to identify the site of cusp formation; while in the molars, these remaining primary enamel knot cells migrate proximally to the future sites of the metacone or metaconid cusps (Aberg *et al.* 2004, Gritli-Linde *et al.* 2002, Lesot *et al.* 1996, Matalova *et al.* 2005, Pispá and Thesleff 2003, Thesleff *et al.* 2001, Tucker and Sharpe 1999). These cusp patterns are later fixed in place by the terminal differentiation of the odontoblasts and the deposition of a mineralised dentine matrix (Thesleff *et al.* 2001).

Concurrent to the removal of the primary enamel knot, differentiation of the pre-odontoblasts and pre-ameloblasts, dental papilla cells and epithelial cells lying directly along the epithelio-mesenchymal interface respectively, is induced in conjunction with multi-layering of the epithelial cells above the pre-ameloblasts (Gritli-Linde *et al.* 2002, Jacob and Veis 2006, Thesleff *et al.* 2001). This multi-layered epithelium forms the stratum intermedium (Gritli-Linde *et al.* 2002, Koyama *et al.* 2001, Lesot *et al.* 1996).

During the bell stage, the enamel organ is distinct from the dental mesenchyme and can be seen as four separate elements: (1) the outer enamel epithelium which is separated from the highly vascularised peridental connective tissue by a basal lamina; (2) the inner enamel epithelium which is the site of ameloblast differentiation; (3) the stratum intermedium which acts in conjunction with the inner enamel epithelium during

amelogenesis; and (4) the stellate reticulum, composed of star-shaped epithelial cells with wide irregular intercellular spaces, which acts to recruit nutrients from the outlying circulation; and to protect the tooth crown during formation (Gritli-Linde *et al.* 2002, Ida-Yonemochi *et al.* 2010, Matthiessen and Romert 1980, Nait Lechguer *et al.* 2008).

The tooth crown is formed in the late-bell stage when tooth development shifts from a phase of morphogenesis to a phase of cytodifferentiation, as the odontoblasts and ameloblasts are terminally differentiated (D'Souza *et al.* 1999, Vaahtokari A, *et al.* 1991). Terminal differentiation of the odontoblasts occurs at the sites corresponding to the future cusp tips and is marked by the withdrawal of the pre-odontoblast from the cell cycle, resulting in cell elongation and polarization, in conjunction with secretion of a predentine mix along the future dentino-enamel junction (D'Souza *et al.* 1999, Tucker and Sharpe 2004, Wang *et al.* 2010). The secretion of the mantle dentine layer induces the terminal differentiation of the ameloblasts, which are shown to elongate, change in cytoskeletal structure and alter in polarity of the nuclei and cell organelles (Gritli-Linde *et al.* 2002). The differentiated ameloblasts then deposit a layer of pre-enamel prisms also referred to as aprismatic enamel (D'Souza *et al.* 1999, Simmer *et al.* 2010).

Concurrent to the terminal differentiation of the odonto- and amelo-blasts, blood vessels from the peridental mesenchyme and mandibular mesenchyme are shown to infiltrate the tooth bell structure. Blood vessels are observed throughout the entire dental pulp (formed from the remainder of the dental papilla cells directly below the odontoblast layer); and are identified perforating the edge of the outer enamel epithelium and invading the stellate reticulum (Arana-Chavez and Massa 2004, D'Souza *et al.* 1999, Wise *et al.* 1990).

As the tooth crown matures, a continuous process of organic matrix secretion coupled with secreted matrix mineralisation occurs in both the odontoblasts and the ameloblasts. In the odontoblasts, the odontoblastic processes secrete collagen which forms structural

cross-linkages with other collagen fibrils; it is within these cross-linkages that mineral formation occurs, leading to the foundation of a mineralisation front (Linde and Goldberg 1993, Smith and Lesot 2001). As the odontoblasts continue to deposit the organic matrix, they move in an apical direction toward the pulp cavity (Arana-Chavez and Massa 2004). Ameloblasts form a mineralisation front by depositing enamel proteins in the extracellular space along the ameloblast cell membrane; these enamel proteins form enamel ribbons which are lengthened in the direction of what remains of the outer enamel epithelium and are thickened by the deposition of amorphous calcium phosphate (Simmer *et al.* 2010, Wise *et al.* 1990). The thickness of these enamel ribbons will determine the hardness of the mature enamel as ribbons are required to interlock to add structural stability to the composite. The amorphous calcium phosphate surrounding the enamel ribbons is then converted to hydroxyapatite and maturation ameloblasts are shown to deposit calcium, phosphate and bicarbonate ions into the organic matrix. This process removes water and neutralises the acidity created in the conversion of calcium phosphate to hydroxyapatite, thus strengthening the structure further (Nait *et al.* 2008, Simmer *et al.* 2010). Carbonic anhydrase is also considered to play a role in neutralising the acidity of hydroxyapatite formation in the maturing enamel as it is known to catalyse the reversible hydration-dehydration reaction of carbon dioxide and bicarbonate, and is not found in the enamel epithelium at any stage prior to that of maturation (Wang *et al.* 2010).

Formation of the tooth crown is completed postnatally and this is followed by the induction of tooth root formation through the apical elongation of cells of the cervical loop, also referred to at this stage as the cervical margin or enamel organ cuff (Fujiwara *et al.* 2008, Grando Mattuella *et al.* 2007, Ida-Yonemochi *et al.* 2005). It was originally hypothesised that the root forms from a bilayer of epithelium of the outer and inner enamel epithelium as the cervical loops mark the junction where these two epithelia

meet. However, it has been shown that there is a cessation of inner enamel epithelium proliferation prior to root development and the bilayer known most commonly as Hertwig's epithelial root sheath is formed from the outer enamel epithelium only (Fujiwara *et al.* 2008). As these cells proliferate apically, the immature root apex is seen to be made up of four distinct zones: (1) the dental sac/follicle which gives rise to the tissue of the periodontium; (2) radicular dental pulp which extends from the coronal portion of the tooth to the apex; (3) the apical dental papilla mesenchyme which is attached externally at the apical diaphragm and is thought to contribute to the periodontium; and (4) the apical cell-rich zone which is comprised of apical dental papilla mesenchyme but is encased within the root sheath at the apical diaphragm (Gritli-Linde *et al.* 2002, Sonoyama *et al.* 2008, Thesleff *et al.* 2001, Thomas 1995, Tziafas and Kodonas 2010). The cementum which forms over the circumpulpal dentine of the root structure is produced in a reaction at the epithelio-mesenchyme junction, as in enamel formation, where the cells of the Hertwig's epithelial root sheath differentiate into functional cementoblasts following odontoblast differentiation (Fong *et al.* 2005, Sonoyama *et al.* 2007).

In the murine model it has been shown that root and periodontal ligament (PDL) formation is completed 4 weeks postnatally (Ida-Yonemochi *et al.* 2005). Tooth eruption through the gingival margin of the mandible or maxilla is then initiated by an infiltration of monocytes into the dental follicle. This infiltration is thought to be caused by the secretion of a monocytic chemoattractant from the stellate reticulum into the capillaries of the dental follicle, initiating monocyte migration into the follicle. Concurrently, there is an increase in the number of osteoclasts present in the surrounding alveolar bone, which facilitates in alveolar bone resorption, constructing a pathway for tooth eruption (Cohen 1994, Sonoyama *et al.* 2007).

This overview of tooth development has been summarised in **Figure 1**.

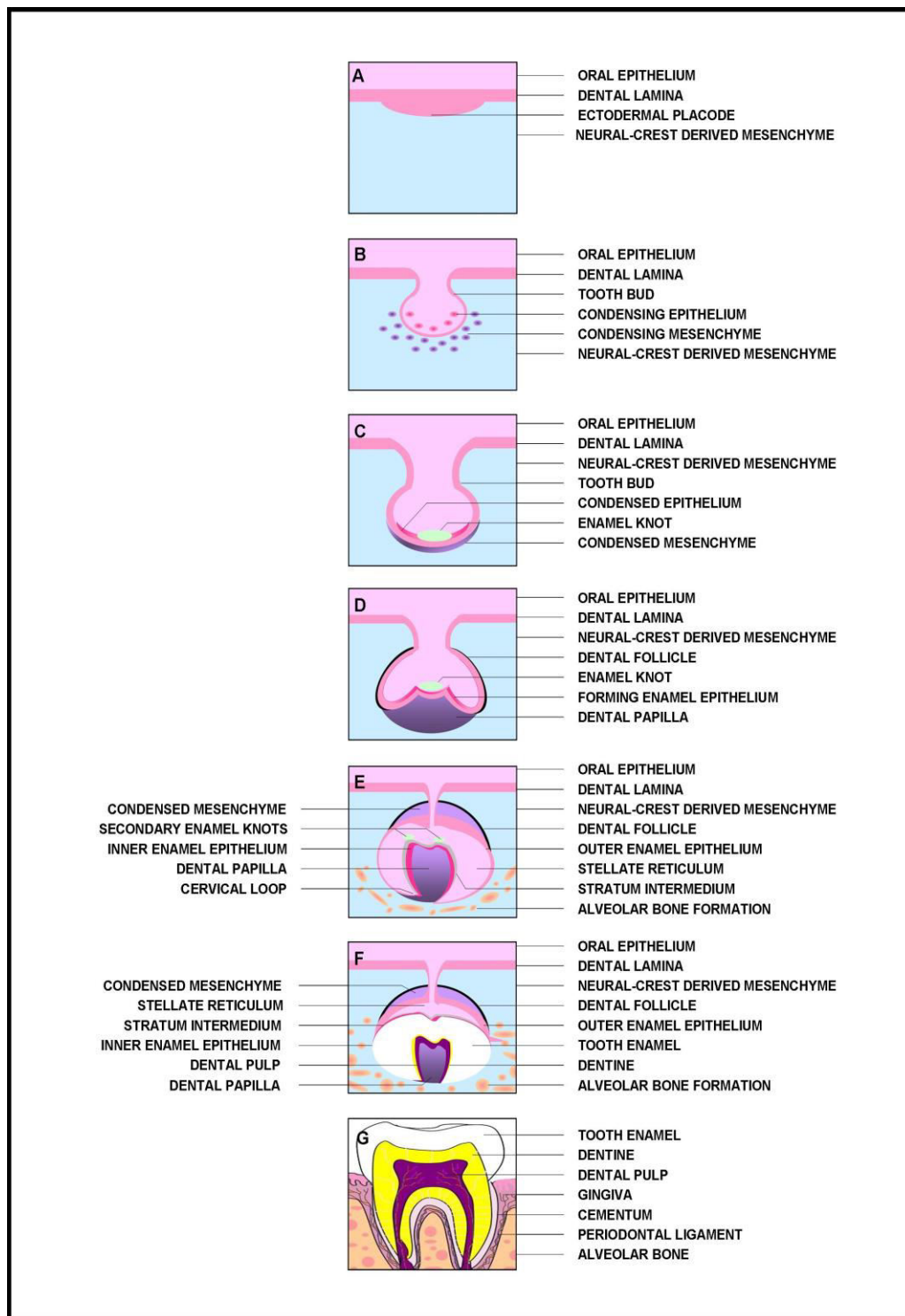


Figure 1 Stages of tooth development

Illustration of the five phases of tooth development: (1) Initiation [A]; (2) Bud Stage [B – Early & C – Late]; (3) Cap Stage [D]; (4) Bell Stage [E – Early & F – Late]; (5) Crown/Maturation [G].

1.1.2 Anatomy of the Dental Pulp

The dental pulp can most straightforwardly be described as having three functional modalities which are directly correlated with its specialised and well-defined morphology. These are: (1) Reparatory – The tissue contributes to the restoration of the encasing calcified tissues through the formation of secondary and tertiary dentine (Goldberg *et al.* 2004, Hosoya and Nakamura 2015, Ricucci *et al.* 2014, Sloan and Smith 2007, Stanley *et al.* 1966,); (2) Sensory – In response to irritation from external factors, e.g. extreme temperature differences (Baldissara *et al.* 1997, Hannig and Bott 1999, Pohto and Scheinin 1958), or internal inflammation (Farges *et al.* 2013), e.g. response to resultant localised infection from caries, endodontal or periodontal disease, patterns of neural activity are induced giving rise to a pain stimulus (Gopinath and Anwar 2014, Jain and Gupta 2013, Narhi *et al.* 2016); and (3) Regulatory – Cellular vitality is strictly maintained through homeostasis in order to sustain tissue integrity and functionality (Smith 2003).

1.1.2.1 Dental Pulp Morphology

The dental pulp anatomically resembles a vascular loose connective tissue encased within rigid enamel, dentine and cementum sheaths; and is developed from the mesenchyme following pre-odontoblast differentiation during odontogenesis (Grando Mattuella *et al.* 2007, Linde and Goldberg 1993). Pulpal development continues throughout the stage of apexogenesis, forming the radicular or root pulp which is clearly distinguishable from that of the coronal portion, pertaining to the crown (Cohen 1994, Thesleff 2006). In addition to being well-defined across the horizontal plane, the pulp can be divided longitudinally into four distinct zones: (1) Odontoblastic zone, (2) Cell-free zone of Weil, (3) Cell-rich zone of Höhl, and (4) Pulp core or pulp proper

(Avery 2006a, Avery 2006b, Avery and Avery 2001, Berkovitz 2002, Cohen 1994, Nanci 2003).

The outermost layer of the pulp, the odontoblastic zone, is comprised primarily of odontoblast cell bodies and lies directly beneath the predentine layer, in which the odontoblastic processes are embedded within the dentinal tubules (Avery 2006, Berkovitz and Holland 2002, Cohen 1994). Capillaries, nerve fibres and dendritic cells are also found within this cell layer although their numbers are few (Cohen 1994, Hargreaves 2002). In the coronal portion of the pulp the odontoblasts are functionally active and maintain a tall columnar shape, while cells within in the mid-radicular region are cuboidal having low-level activity. The odontoblasts located in the most apical portion of the root are quiescent and appear as a flattened layer at the apical foramen (Berkovitz and Holland 2002, Cohen 1994, Nanci 2003).

Subjacent to the odontoblastic zone in the coronal pulp, lies a narrow region relatively free from cells. This cell-free zone, also referred to as Weil's basal layer, has been demonstrated to be traversed with capillary vessels, unmyelinated nerve fibres and the cellular processes of fibroblasts of the cell-rich zone (Avery 2006, Cohen 1994, Hargreaves 2002, Linde and Goldberg 1993). The cell-free zone has been shown to disappear in pulps where the odontoblastic layer is disrupted, such as in teeth which have been avulsed or those treated with an orthodontic force, and is also absent from the maturing pulps of young teeth (Breivik 1981, Cohen 1994, Santamaria *et al.* 2007).

Directly beneath the cell-free zone lies the cell-rich zone of Höhl. This cell-rich layer is composed from a collection of fibroblasts, undifferentiated mesenchymal progenitor cells, macrophages and lymphocytes; and is most prominent in the coronal portion of the tissue (Cohen 1994, Hargreaves 2002, Jontell *et al.* 1998, Nanci 2003, Tziafas 2010). Fibroblasts are responsible for the production and secretion of proteoglycans, fibronectin and other extracellular matrix components such as type I and type III

collagens, which are fundamental components in the support of the vasculature (Avery and Avery 2001, Cohen 1994). They are particularly responsible for the maintenance and turnover of the extracellular matrix, as they are capable of the tissue-specific production and degradation of collagen fibrils (Avery 2006, Avery and Avery 2001, Cohen 1994).

The connective tissue of the pulp proper or pulp core makes up most of the mass of the dental pulp and is composed of a gelatinous extracellular matrix constituting: blood and lymph vessels; nerve trunks; glycoproteins; ground substance; hydrophilic glycosaminoglycans including heparin sulphate and hyaluronic acid; fibroblasts and immunocompetent cells including macrophages, B-lymphocytes and T-lymphocytes (Avery and Avery 2001, Berkovitz and Holland 2002). The combination of collagen, proteoglycans and glycoproteins within the extracellular matrix enable the pulp core to maintain large volumes of water, giving the non-compressible tissue added physical strength in its unique non-compliant environment (Harlamb and Messer 1996).

1.1.2.2 Dental Pulp Vasculature

Development of the vasculature of the dental pulp is initiated at the bell stage of odontogenesis where blood vessels from the peridental and mandibular mesenchyme infiltrate the structure (Arana-Chavez and Massa 2004, D'Souza *et al.* 1999, Wise *et al.* 1990). It is known that pulpal vasculature formation coincides with the differentiation of odontoblasts and the formation of dentine, as odontoblasts require a rapid and sufficient supply of raw materials for the production and subsequent calcification of the dentine matrix (Yoshida and Ohshima 1996).

Initially, the vasculature is formed from a coarse network of thick capillaries, which increases in density and decreases in vessel diameter at the onset of odontoblast differentiation (Yoshida and Ohshima 1996). At this stage of development the

capillaries are located within the inner region of the dental pulp and have no contact with the odontoblasts (Yoshida and Ohshima 1996). As predentine formation is induced, the vascular network increases in density and the external surface of the capillaries becomes undulated, while vessels begin to invade the odontoblastic layer (Yoshida and Ohshima 1996). Dentine formation continues as the mineralisation front pushes the odontoblasts apically and distally toward the pulp core, at this stage vessels are shown to have a flat and dense external surface; and are seen penetrating the predentine layer (Yoshida and Ohshima 1996). At the completion of crown formation, odontoblastic activity is reduced and the peripheral capillaries of the coronal pulp retreat from the predentine layer to the odontoblastic layer (Yoshida and Ohshima 1996).

In the fully formed tooth, the vasculature of the dental pulp is characterised as a complex, isolated network of precapillary arterioles, capillaries, postcapillary venules and muscular venules (Digka *et al.* 2006, Rodd 2005). Blood and lymph vessels in conjunction with nerve fibre bundles enter the tooth through the apical and accessory foramina; and course along the radicular root axis toward the chamber of the coronal pulp (Berkovitz and Holland 2002, Hargreaves 2002, Rodd and Boissonade 2005, Vandevska-Radunovic *et al.* 1997). In the coronal pulp, the capillary plexus expands and presents as three distinct layers: (1) the terminal capillary network which lies parallel and in close proximity to the predentine; (2) the central capillary network which lies perpendicular to the pulp surface; and (3) the venular network which lies parallel to the odontoblastic surface and acts as a source of drainage in the tissue (Rodd 2005, Yoshida and Ohshima 1996, Vandevska-Radunovic *et al.* 1997). In order to ensure that sufficient oxygen reaches the fibroblasts and odontoblasts, capillary loops are formed within the subodontoblastic region (Avery 2006.).

Myelinated and unmyelinated sensory and sympathetic nerve fibres within the pulp are thought to play a specific role in the regulation of tissue pressure and microcirculation

(Hargreaves 2002, Okamura *et al.* 1994, Rodd and Boissonade 2005, Zhang *et al.* 1998). In a study investigating the pulpal response to caries, a significant increase in vascularity was observed within the pulpal horns; however, this increase did not pertain to vessel number but rather to vessel size (Rodd and Boissonade 2005). As the highest concentration of neural elements has been localised to the pulp horns, and terminal axons from unmyelinated nerves are located exclusively adjacent to the outermost cells of the smooth-muscle layer of arterioles; it can be concluded that vasomotor nerves regulate pulpal microcirculation through the contraction or relaxation of sphincter-like vessels (Hall and Freer 1998, Hargreaves 2002, Okamura *et al.* 1994, Zhang *et al.* 1998).

1.1.3 Orthodontic Tooth Movement

Orthodontic tooth movement is characterised by the remodelling of dental and paradental tissues resulting in translocation and alteration of tooth position (Krishnan and Davidovitch 2006). Application of orthodontic force has been demonstrated to result in areas of tension and compression, within the supporting alveolar bone, in conjunction with injury to the periodontium and associated vascular network (Davidovitch *et al.* 1988, Mostafa *et al.* 1991).

1.1.3.1 Mechanisms of Orthodontic Tooth Movement

Orthodontic tooth movement has been postulated to occur by one of two processes: (1) pressure-tension theory (Oppenheim 2007) or (2) bone bending/bioelectric theory (Baumrind 1969, Davidovitch *et al.* 1980a, Davidovitch *et al.* 1980b, Zengo *et al.* 1974). In both systems it has been noted that tooth movement (through the processes of bone, gingival and PDL remodelling) occurs in distinct phases, which from hereon will be referred to as the: (a) initial, (b) lag, (c) acceleration and (d) linear phases (Pilon *et al.* 1996). In the initial phase of tooth movement which can last anywhere from 24 to

48 hr (Krishnan and Davidovitch 2006, Meeran 2013, Zainal Ariffin *et al.* 2011), rapid displacement of the tooth in the periodontal ligament (PDL) space and tooth socket occurs, resulting in damage to the cells in the PDL at the “pressure” side of the tooth, in the direction of tooth movement (Dorow *et al.* 2002, Henneman *et al.* 2008, Mavragani *et al.* 2004, van Driel *et al.* 2000). The tooth then enters the lag phase, where movement is halted until necrotic tissues are removed by phagocytosis in a process that can last up to 20 days (Von Bohl *et al.* 2004, Zainal Ariffin *et al.* 2011). Movement is then accelerated (acceleration phase) through the alveolar bone in a continuous cycle of bone resorption and osteoid deposition until an internal biologically limiting rate is reached where the rate of movement is at a constant and is no longer dependent upon the magnitude of force which has been applied (Pilon *et al.* 1996). This is referred to as the linear phase of orthodontic tooth movement.

It has been shown that during the initial and lag phases of tooth movement that a compression of the PDL vasculature at the “pressure” side, results in localised ischemia and hypoxia, leakage of blood constituents into the surrounding tissue, tissue hyalinization, and cell death (Kitase *et al.* 2009, Miyagawa *et al.* 2009, Ren *et al.* 2008). While at the “tension” side, in the direction opposite to tooth movement, there is an increase in vessel formation which has been shown to aid in the alveolar bone remodelling process (Ren *et al.* 2008). It has been postulated that the circulatory damage which occurs in the vasculature at the “pressure” side not only results in localised hypoxia in the PDL, but that this has a knock-on effect on the highly sensitive tissue contained within the pulp chamber of the tooth (Romer *et al.* 2014, Sano *et al.* 2002).

1.1.3.2 *Response of the Human Dental Pulp to Orthodontic Tooth Movement*

A number of investigations have been performed in order to determine the physiological and pathological response of the periodontium and dental pulp to orthodontic force application (Abi-Ramia *et al.* 2010, Grunheid *et al.* 2007, Han *et al.* 2010, Kvinnsland *et al.* 1989, Nakamura *et al.* 2008, Nakanishi *et al.* 2004, Toyono *et al.* 1997, Tripuwabhrut *et al.* 2010, Uematsu *et al.* 1996, Von Bohl *et al.* 2004, Von Bohl *et al.* 2016). However, few of these studies have focussed on the response of these tissues within humans; despite the clinical application of these practises being carried out in humans. The small number of these studies which have been performed in the human draws attention to the need for further investigation into the effects of routine clinical treatment; it also highlights the issues surrounding standardisation of experimental protocols, as groups which have investigated a single issue, e.g. force intensity, will have to extrapolate findings from a model that often responds very differently to the target organism (Noda *et al.* 2009, Sano *et al.* 2002).

It has been established within the literature, that following force application, human dental pulps will respond by: (a) vacuolisation (Han *et al.* 2013, Ramazanzadeh *et al.* 2009, Stenvik and Mjor 1970), (b) nodule formation (Lazzaretti *et al.* 2014) or (c) hypoxia and alteration to the pulpal blood flow (Hamersky *et al.* 1980, Mostafa *et al.* 1991, Sano *et al.* 2002). Pulpal blood flow is a topic of particular interest as hypoxia could result in necrosis of the tissue. Should hypoxia occur, it is anticipated that angiogenesis will occur in order to reoxygenate the tissue.

Angiogenesis has been outlined as a potential response of the pulp to orthodontic force application as several angiogenic growth factors have been found to be secreted from the human dental pulp following a fortnight of treatment (Derringer *et al.* 1996, Derringer and Linden 1998, Derringer and Linden 2003, Derringer and Linden 2004, Derringer and Linden 2007).

1.1.4 Angiogenesis

Angiogenesis is the process of new vessel formation from a pre-existing capillary plexus (Brown *et al.* 1992, Li *et al.* 2003, Yano *et al.* 2003) and is known to occur in the embryo during development following vasculogenesis and in the adult as a result of localised hypoxia, reaction to tissue damage or an imbalance of regulatory cytokines (Carmeliet 2000, Risau 1996, Risau 1997). This complex signalling cascade has been shown to occur by one process or by two processes concurrently: sprouting angiogenesis (Carmeliet 2000, Folkman and D'Amore 1996) and intussusceptive angiogenesis (Burri *et al.* 2004, Djonov *et al.* 2000); and is tightly regulated by a large number of cytokines, chemokines and growth factors.

1.1.4.1 Intussusceptive Angiogenesis

Intussusceptive angiogenesis has been defined by Burri *et al.* (2004) as the 'further development of a capillary network through the insertion of transcapillary pillars'. Although originally described by Short (1950), little is known about its functional mechanisms as sprouting angiogenesis has remained at the forefront of current research investigations. It is considered that like sprouting angiogenesis, intussusception occurs in a series of well-defined stages and sub-stages which results in the formation of new vessels from the pre-existing vascular plexus; and it has been shown to play a role in both the embryo (following vasculogenesis) (Risau 1997) and in the adult (Burri and Djonov 2002) in a response to inflammation or chronic hypoxic exposure (De Spiegelaere *et al.* 2012, Styp-Rekowska *et al.* 2011).

Intussusceptive angiogenesis has been determined to occur in three stages: (a) intussusceptive microvascular growth, (b) intussusceptive arborisation and (c) intussusceptive branching remodelling (Makanya *et al.* 2009). In the primary stage of intussusceptive microvascular growth, a number of well defined sub-stages have

been characterised. Angiogenesis is initiated when the outer wall of two proximal capillaries touch and form an interendothelial transluminal bridge (Burri *et al.* 2004, Djonov *et al.* 2000, Kurz *et al.* 2003). Following the formation of the bridge, the circumference of the contact zone is sealed by junctional complexes in order to prevent vascular leakage (Burri and Djonov 2002, De Spiegelaere *et al.* 2012). The intercellular junctions of the endothelial bilayer are then reorganised and a central perforation is formed (Djonov *et al.* 2000, Kurz *et al.* 2003). The endothelial cells of the contact zone expand and flatten out to form transcapillary tissue pillars, which are then invaded by cytoplasmic extensions of myofibroblasts, pericytes and interstitial fibers from the external surface of the endothelium (De Spiegelaere *et al.* 2012, Djonov *et al.* 2000, Kurz *et al.* 2003). Pillar diameter is then allowed to increase and they are stabilised further (Djonov *et al.* 2000, Kurz *et al.* 2003). The opposing walls at the centre of the perforation then meet and form a contact zone and through the process of cellular reorganisation, longitudinal vessels will form; and the vasculature will have the phenotype of a large capillary mesh (Djonov *et al.* 2000).

Following initial intussusceptive microvascular growth, angiogenesis moves into the second stage: intussusceptive arborisation. Not much is known about the sub-stages of this phase of angiogenesis; but it is known that the neovessels adapt into a typical vascular tree pattern where arterioles, venules and capillaries are clearly identifiable (Djonov *et al.* 2000, Makanya *et al.* 2009). In the final stage of angiogenesis, intussusceptive branching remodelling, the neovessels are ‘pruned’ by the formation a number of occluding endothelial cell – endothelial cell contact zones in order to remodel the vasculature for current perfusion requirements (Makanya *et al.* 2009).

Unlike sprouting angiogenesis, intussusception does not rely on increased endothelial cell proliferation, basement membrane degradation or on cell signalling from alternate tissues (Burri and Djonov 2002, De Spiegelaere *et al.* 2012); it is however moderated by

a number of angiogenic growth factors, cytokines and chemokines (De Spiegelaere *et al.* 2012, Burri and Djonov 2002, Styp-Rekowska *et al.* 2011). These factors are important to consider as their expression may act as a biomarker of the mechanism of angiogenesis that is occurring in the tissues. This is particularly true for the growth factor vascular endothelial growth factor alpha (VEGFA) which is known to be a leading protagonist in the sprouting angiogenic cascade but has been shown to have little effect or control over intussusception (Burri and Djonov 2002).

1.1.4.2 Sprouting Angiogenesis

Sprouting angiogenesis can be defined as the outgrowth of endothelial sprouts, composed of tip and stalk cells, from the existing vasculature towards an angiogenic stimulus (Gerhardt 2000-2013, Gerhardt *et al.* 2003, Heck *et al.* 2015). Originally characterised by Ausprank and Folkman (1977) in the rabbit cornea, it was established that neovascularisation of the eye by sprouting angiogenesis occurred in a number of well-defined stages. We have defined these stages according to the literature as: (a) Receipt of stimulus from signalling tissue or cells (Kilarski 2005), (b) Localised degradation of the endothelial basement membrane of the responding vasculature (Brooks *et al.* 1996, Silletti *et al.* 2001), (c) Reorganisation of the inter-endothelial junctions and concurrent selection of tip cells (Bentley *et al.* 2008, Jakobsson *et al.* 2010), (d) Degradation of the surrounding matrix and concurrent sprout extension. (Brooks *et al.* 1996, Silletti *et al.* 2001), (e) Guided migration of the vascular sprout and simultaneous stalk cell proliferation (Gerhardt *et al.* 2003, Phng *et al.* 2013), (f) Lumen formation by apical membrane invagination. (Gebala *et al.* 2016) and (g) Anastomosis of the sprout with the signalling endothelium accompanied by new basement membrane synthesis with pericyte recruitment. (Bergers and Song 2005, Bondjers *et al.* 2003, Song *et al.* 2012).

Due to the complex nature of this angiogenic mechanism, where its stages are regulated by the varied expression of a vast number of cytokines, chemokines and growth factors at any one time, a full and comprehensive review of the signalling cascade is implausible. It had previously been postulated by researchers that during each stage of sprouting angiogenesis, there was a set regulated increase or decrease in a specific growth factor and that this increase or decrease was restricted to act upon a particular cell type. However, the new emerging model of angiogenesis is that of a bee colony, where individual endothelial cells hold differing roles, express differing phenotypes and respond to angiogenic cues in an individualistic manner; and that depending upon the angiogenic cues, cells may alter their role and phenotypes at any time (Chappell *et al.* 2011). In conjunction with this new model of cell behaviours, it has been highlighted that the concept of a single increase or decrease in expression of a protein of interest will not fully replicate the signalling mechanisms *in vivo.*; and that should all factors not be activated in a coordinated and complementary manner, functional vessels will not form (Yancopoulos *et al.* 2000). We support these statements with the findings of a review of the sprouting angiogenesis protagonist, VEGFA, where it has been shown that during angiogenesis, extracellular VEGFA is controlled at the pre-transcriptional, splicing, cell surface retention, protein uptake and degradation stages; and that the complex regulation of this single gene will result in an extracellular gradient which controls proper vascular patterning (Gerhardt 2000-2013. Gerhardt *et al.* 2003). As VEGFA is only a single gene within this highly complex signalling cascade, it is clear to see that any alteration to the concerted up- and down- regulation of mRNA in conjunction with increased or decreased protein expression could result in a pathological phenotype.

1.1.5 Hypoxia and Angiogenesis

Hypoxia has been defined as the state where oxygen concentrations within a tissue or cell have been reduced below physiological levels, despite the presence of adequate perfusion by blood (Dorland 2007). It can occur continuously or intermittently whilst ranging in severity from an acute to a chronic condition, depending on the physiological or pathological cause. Hypoxia is mediated by the stabilisation of the mRNA of the transcription factor hypoxia inducible factor-1, alpha (HIF1A) and is regulated homeostatically by oxygen dependent hydroxylation of the oxygen-dependent degradation (ODD) domain of the HIF1A protein by prolylhydroxylase (PHD) enzymes (Ivan *et al.* 2001, Jaakkola *et al.* 2001); or polyubiquitylation of the von Hippel-Lindau tumour suppressor (pVHL) E3 ligase complex (Jaakkola *et al.* 2001, Maxwell *et al.* 1999, Semenza 2003). Should the HIF1A transcript become stabilised by (1) decreased oxygen concentration and an absence of oxygen-dependent hydroxylation, (2) nitrosylation of the oxygen-dependent domain of the HIF1A subunit by free radicals (Li *et al.* 2007) or (3) up-regulation of growth factors which inhibit PHD enzymes necessary for hydroxylation (Dengler *et al.* 2014, McMahon *et al.* 2006); angiogenesis, and the concordant up-regulation of angiogenesis-specific growth factor mRNA and increased protein transcription occurs in order to regulate oxygen tension (Krock *et al.* 2011, Pugh and Ratcliffe 2003, Semenza 2001).

Angiogenesis has been shown to be induced in a number of tissues in response to hypoxia (Brogi *et al.* 1994, Liu *et al.* 2013, Pham *et al.* 2002, Raja *et al.* 2014); and this is mediated through the hypoxic-induction of VEGFA by HIF1A binding to the HIF-related binding site located within the 5'-flanking region of the VEGFA gene (Abraham *et al.* 2004, Flamme *et al.* 1997). Activated VEGFA then binds to the kinase insert domain receptor (a type III receptor tyrosine kinase) (KDR), a predominantly vascular endothelial tyrosine kinase (Millauer *et al.* 1993), to induce endothelial cell

proliferation. This proliferation is then linked to an increase in expression of growth factors and cytokines as the complex processes of angiogenesis are initiated. As previously discussed, VEGFA has been shown to have little influence on intussusceptive angiogenesis as this mechanism of angiogenesis is not driven by external signalling. Instead, VEGFA will play a significant role in the proliferation of the endothelial sprout during sprouting angiogenesis.

While it is commonly accepted that hypoxia and angiogenesis work hand in hand, in a supply and demand relationship, this hypothesis is now being called into question, as a strong link has been established between inflammation and hypoxia (Moeller *et al.* 2004). It is now a consideration that inflammation will induce HIF1A expression and it is rather inflammation that induces angiogenesis, as hypoxia alone is insufficient.

1.1.6 Hypoxic and Angiogenic Growth Factors in the Human Dental Pulp

The human dental pulp is a known source of regulatory cytokines, chemokines and growth factors and of these factors, a number are associated with both the angiogenic and hypoxic signalling cascades. Below are outlined a number of investigations which have determined the presence of factors associated with both the angiogenic and hypoxic signalling cascades:

1. In an attempt to identify a pathological intervention for bone healing in distraction osteogenesis, Fujio *et al.* (2015) established that the culture media from human dental pulp cells which had been exposed to hypoxia contained a number of factors that promote bone healing; these factors include angiopoietin 1 (ANGPT1) and VEGFA.
2. Odontoblast differentiation was studied by Kim *et al.* (2010) with the view to induce mineralisation for reparative dentistry. They established that fibroblast growth factor 2 (basic) (FGF2) plays a role as both (a) an inducer of

odontoblastic differentiation and (b) a chemokine regulator within human dental pulp cells.

3. Hypoxia was shown to induce the expression of HIF1A and VEGFA in human dental pulp stem cells and human dental pulp fibroblasts when cells were examined with respect to identifying a solution to dental trauma involving the luxation or avulsion of the tooth (Aranha *et al.* 2010).
4. As dental pulp surface adhesion has yet to be characterised fully, so as better to understand the physiology of this tissue, Zhu *et al.* (1998) performed a number of experiments in order to determine the integrin expression patterns in human dental pulp cells cultured from third molar teeth. They established that a number of integrins were expressed by the tissue, some of which include integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51) (ITGAV) and integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61) (ITGB3).
5. In addition to establishing that VEGFA is expressed within the human dental pulp, it has been shown that its receptor KDR is expressed in the vasculature of the human dental pulps (Virtej *et al.* 2013).
6. In an attempt to confirm the effectiveness of New Zealand Fetal Bovine Serum in comparison to Conventional Fetal Bovine Serum, in order to continue the promotion of the use of serum in cell culture protocols; investigators established that human dental pulp stem cells grown in serum expressed higher levels of mRNA for platelet-derived growth factor alpha polypeptide (PDGFA) and VEGFA under their preferred experimental conditions (Spina *et al.* 2016).
7. Orthodontic traction was investigated to determine whether there would be any long-lasting effects on the tissue. Nitric oxide synthase 2, inducible (NOS2), a pro-inflammatory chemokine was found to be present in both the control and the treated teeth and was explained as a response to the extraction process. Matrix

metallopeptidase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase) (MMP2) expression was shown to significantly decrease over the duration of the treatment and this was interpreted to correlate with an impediment of the pulp to regenerate and restore proper function (Leone *et al.* 2009).

8. Cell adhesion molecules in the human dental pulp endothelial cells were assessed in order to determine the effect of reparative crowning with adhesive on the pulps' inflammatory response. It was established that a number of cell adhesion molecules including selectin E (SELE) were present in both the control and treated teeth; and that the application of a crowning adhesive resulted in pulpal inflammation (Bagis *et al.* 2007).
9. Odontoblastic differentiation in tooth repair by mineralisation was investigated by Yongchaitrakul and Pavasant (2007) in order to establish the effect of transforming growth factor, beta 1 (TGFB1) on nerve growth factor (NGF) in injured dental pulps; as NGF promotes survival and repair of nervous tissues. They identified that TGFB in conjunction with its receptor, transforming growth factor, beta receptor 1 (TGFB1) will induce NGF in human dental pulp cells, suggestion that TGFB1 may play a role in tooth repair following injury.

Chapter II

Materials and Methods

MATERIALS

2.1 Human Dental Pulp Tissue

Following informed parental and patient consent, human dental pulps were obtained from healthy first and second premolar teeth extracted from volunteers aged between 12 – 16 years. All patients included in the study required extraction of a minimum of 2 contralateral premolar teeth from a paired dental position (i.e. first upper left premolar and first upper right premolar), in conjunction with fixed appliance orthodontic treatment. Ethical approval was granted by the Research Ethics Committee at Guy's Hospital and King's College Hospital.

METHODS

2.2 Orthodontic Force Application and Dental Pulp Isolation

In each experimental chapter, unless otherwise specified, contralateral pairs of control and orthodontically treated teeth were used to assess mRNA and protein expression in the angiogenic response of human dental pulp to orthodontic force application. Duration of force application to the teeth prior to extraction ranged from no treatment to 2 weeks of treatment and specific details are provided in the materials and methods section of the experimental chapters.

In every patient, the oral cavity was divided along the mid-sagittal plane in order to assign a 'control' sample that would forego orthodontic force application; and a contralateral 'treated' sample which would experience a force in the range of 0.5 – 1 N for the duration of treatment. Fixed appliance straight wire orthodontic brackets (Roth 0.022 inch, 3M Unitek, California, USA) were direct bonded (Transbond, 3M Unitek,

California, USA) to the upper and/or lower teeth from second premolar to second premolar, except the assigned 'control' teeth. Orthodontic bands were cemented (Ketac, 3M ESPE, Seefeld, Germany) onto all first permanent molar teeth. Test tooth position was carefully assessed and brackets were bonded in a position (offset if necessary) so that the archwires (0.016 inch nickel-titanium, TP Orthodontics Inc., Leeds, UK) placed gave the required force of 0.5 – 1 N in a mesial and extrusive direction.

Following the required period of force application, brackets were removed from the premolar teeth assigned as 'treated' teeth. The 'control' and contralateral 'treated' premolar teeth were extracted under local anaesthesia, placed in individual tubes containing 20 ml of cold sterile saline solution, and sectioned immediately with a high-speed water-cooled diamond bur through the buccolingual aspect. Dental pulps with a mean mass of ± 0.05 g ($n = 5$) were removed from the pulp chamber with sterile blunt instruments. Isolated pulps were then: (a) individually placed in a sterile 1.5 ml centrifuge tube and snap-frozen in liquid nitrogen before transferring to -70°C for permanent storage; or (b) immersed separately in 500 μl RNALater™ (Catalogue Number (Cat. No.) AM7021) (Ambion Inc., Warrington, UK), an RNA preservation solution, kept at 4°C for 8 hr and transferred to -20°C for permanent storage.

All orthodontic procedures and dental pulp isolations, following tooth extraction, were performed by Dr K. A. Derringer, Dental Institute, King's College London at Guy's Hospital and King's College Hospital.

2.3 RNA Isolation

Unless stated otherwise, $\text{RNA}_{\text{total}}$ was isolated from individual pulp samples using a modified phenol:chloroform extraction protocol that had been optimised in collaboration with Dr N. Silver, Dental Institute, King's College London.

Pulp samples were ground in a 7 ml glass Dounce tissue homogeniser (Wheaton Science Products from VWR, Poole, UK) containing 600 µl of the RNA stabilisation solution RNA-Bee™ (Cat. No. CS-104B or CS-105B or CS-501B) (AMS Biotechnology, Abingdon, UK) and homogenates were transferred to sterile centrifuge tubes. 60 µl of chloroform (Cat. No. C7559) (Sigma-Aldrich, Poole, UK) was added to the homogenate and samples were centrifuged at 4 °C for 15 min at 9,000 g. Supernatants were collected in fresh tubes and a single volume of 70 % ethanol was added. Samples were inverted repeatedly and transferred to spin columns and RNA_{total} was extracted using the RNEasy Mini Kit (Cat. No. 74104 or 74106) (Qiagen Ltd, Crawley, UK) according to manufacturer's instructions. Isolated RNA_{total} was suspended in a final volume of 30 µl nuclease-free water (Cat. No. AM9938) (Ambion Inc., Warrington, UK) and stored at -70 °C.

2.4 RNA Quantification

Unless specified otherwise, the purity and concentration (ng/µl) of RNA_{total} for each sample was determined by calculating the A₂₆₀:A₂₈₀ and A₂₆₀:A₂₃₀ ratios using the NanoDrop™ 1000 spectrophotometer (Now replaced by Cat. No. ND-2000, NanoDrop™ 2000 spectrophotometer) (ThermoFisher Scientific, Nottingham, UK), according to manufacturer's instructions. RNA_{total} was then quantified by fluorescence with the Quant-iT™ Ribogreen® RNA Assay Kit (Cat. No. R11490) (Molecular Probes, Invitrogen Ltd., Paisley, UK), according to the manufacturer's protocol.

2.5 Reverse Transcription

The general procedure for reverse transcription of RNA_{total} to cDNA is as follows; details of alternate protocols are given in the materials and methods section of the experimental chapters.

50 ng RNA_{total} was converted to cDNA using the WT-Ovation™ Pico RNA Amplification System (Now replaced by Cat. No. 3302, Ovation Pico WTA System V2) (NuGen Technologies, Inc., California, USA) according to the manufacturer's protocol. cDNA was then purified using the DNA Clean and Concentrator 25™ kit (Cat. No. D4033 or D4034) (Zymo Research Corporation, California, USA) according to manufacturer's instructions; and quantified with the NanoDrop™ 1000 spectrophotometer.

2.6 Quantitative real-time reverse-transcription Polymerase Chain Reaction (RT-qPCR)

The general procedure for RT-qPCR analysis of mRNA expression is as follows; details of alternate protocols are given in the materials and methods section of the experimental chapters along with specific primer sequences, efficiencies and melting points.

RT-qPCR was performed on 20 µl reactions set up manually in 0.1 ml strip tubes (Cat. No. 981103) (Qiagen Ltd., Crawley, UK): 5 µl cDNA (2 ng/µl), 1 µl forward and reverse primer mix (6 µM) (designed and optimised by PrimerDesign Ltd., Southampton, UK), 10 µl Precision Mastermix with SYBR Green (Now replaced by Cat. No. PrecisionPlus-SY, PrecisionPlus™ Mastermix) (PrimerDesign Ltd., Southampton, UK) and 4 µl RNase/DNase-free water (delivered in conjunction with the primers, no individual Cat. No.) (PrimerDesign Ltd., Southampton, UK) with the Corbett Rotor-Gene™ 6000 (No Cat. No. available but item is still available from Qiagen) (Corbett Life Science, Qiagen Ltd., Crawley, UK). Amplifications were performed with an initial template denaturation step at 95 °C for 10 min followed by 50 cycles of denaturation at 95 °C for 15 sec; combined primer annealing/extension at 60 °C for 60 sec; and data acquisition.

A disassociation protocol was performed from 72 °C to 95 °C at 1 °C increments in order to determine the presence of primer-dimer formation or other spurious binding products.

2.7 Statistical Analysis of RT-qPCR Data

RT-qPCR data captured by the Corbett Rotor-Gene™ 6000 and viewed with Corbett Rotor-Gene™ 6000 Application Software, version 1.7 (Build 87) was analysed using the paired Student's t-Test and Relative Expression Software Tool (REST©) 2009 Software (developed by Prof. M. Pfaffl, Technical University Munich, Germany and Qiagen Ltd., Crawley, UK; <http://www.gene-quantification.com/rest-2009.html>).

Chapter III

Optimising Protocols for the Analysis of Human Dental Pulp at the Molecular Level

3.1 Introduction

Current protocols for molecular analysis of gene expression in the human dental pulp are extensively varied (Buchaille *et al.* 2000, Huang *et al.* 2006, Lee *et al.* 2013, McLachlan *et al.* 2005, Paakkonen and Tjaderhane 2010, Park *et al.* 2014, Virtej *et al.* 2013). Tissues are either collected after administration of experimental treatment; or are extracted from untreated teeth and then moved into a culture system for creating cell-type specific cell-lines or for explant culture. RNA_{total} is isolated from cell-lines or explants by a number of recognised methodologies, quantified and then processed for *in situ* hybridisation, suppression subtractive hybridization or microarray analysis; or reverse-transcribed for semi-quantitative RT-PCR or RT-qPCR analysis (Bartlett 2002, Buchaille *et al.* 2000, Felaco *et al.* 2000, Lee *et al.* 2013, Matsushita *et al.* 2000, Shimabukuro *et al.* 2005, Sloan *et al.* 2001, Staquet *et al.* 2006, Ueda *et al.* 2001, Virtej *et al.* 2013).

RT-qPCR is a well established and highly sensitive technique for high-throughput analysis of gene expression profiles and quantification of mRNA; and has proven to be particularly useful for expression analysis of mRNA transcribed in low abundance (Bustin 2000, Sanders *et al.* 2013), in samples with small amounts (ng) of isolated RNA_{total} (Cremer *et al.* 1997) and in limited clinical samples, such as laser-captured tumour tissue or isolated single cells etc (Glockner *et al.* 2000, Saliba *et al.* 2014). While the technique is widely used, there are a number of questions that have been raised about multiple factors, which are frequently overlooked; and their effects on the reliability of RT-qPCR data (Bustin 2002, Bustin 2010, Bustin and Nolan 2004, Bustin *et al.* 2009, Dheda *et al.* 2004). These factors may be introduced at multiple stages of the experimental protocol and include: poor sample preservation (Bustin and Nolan 2004) inaccurate quantification (Huggett *et al.* 2005); RNA integrity (Fleige and Pfaffl

2006, Imbeaud *et al.* 2005); inefficiency in the reverse-transcription step (Bustin *et al.* 2005, Dheda K, *et al.* 2004, Stahlberg *et al.* 2004); manual manipulation of reaction thresholds and therefore quantification points (Bustin 2004, Vandesompele *et al.* 2009); and inappropriate normalisation of gene expression data (Pfaffl 2001, Pfaffl *et al.* 2004, Vandesompele and Kubista 2009). Although it is believed that there is no absolute way to control for disturbances in gene expression quantification (Pfaffl *et al.* 2004), limitation of experimental variability by optimisation of preservation, extraction, quantification and reverse-transcription protocols followed by normalisation against the total amount of input RNA_{total} (ng) or against a stable internal control, to limit the influence of sample-to-sample and run-to-run variability, is considered to be the paragon approach to RT-qPCR (Bustin 2002).

For the internal control, commonly referred to as a housekeeping or reference gene, to be considered as stable it should be expressed constitutively and in abundance in the tissue of interest, showing little variation in expression between the physiological or control state and states induced under different experimental conditions (Bar *et al.* 2009, Coulson *et al.* 2000). A number of studies have been carried out in order to identify a single “universal” reference gene for normalising human gene expression in all tissues (de Kok 2005, Eisenberg and Levanon 2003, Hsiao *et al.* 2001, Warrington *et al.* 2000); nevertheless, attempts to date have been unsuccessful and have resulted instead in the compilation of three lists of human reference genes, available for public access (Eisenberg and Levanon 2003, Hsiao *et al.* 2001, Warrington *et al.* 2000).

In a number of studies, generic reference genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta actin (ACTB) have been shown to be susceptible to regulation by experimental and pathological conditions; and to vary substantially between the control and treated/diseased samples of a single tissue type as a result (Goidin *et al.* 2001, Huggett *et al.* 2005, Revillion *et al.* 2000, Selvey *et al.* 2001,

Sirover 1997, Suzuki *et al.* 2000, Thellin *et al.* 1999, Zhong and Simons 1999). These findings have been the driving force behind a recent surge of investigations attempting to identify an appropriate reference gene for a given experimental system; where it is often concluded that in a complex experiment, evaluating multiple manipulations of a single tissue type, no single suitable reference gene can be identified and normalisation against the geometric mean expression of multiple reference genes is required (Coulson *et al.* 2008, Davoren *et al.* 2008, Fink *et al.* 2008, Gubern *et al.* 2009, Perez *et al.* 2007, Pombo-Suarez *et al.* 2008, Rhinn *et al.* 2008, Stern-Straeter *et al.* 2009, Valente *et al.* 2009).

GAPDH and ACTB have previously been used for normalising data in RT-qPCR studies of the human dental pulp (Dissanayaka *et al.* 2015, Dong *et al.* 2013, Gronthos *et al.* 2002, Liu *et al.* 2007, McLachlan *et al.* 2005) but, as no rationale has been given to substantiate the use of these two generic reference genes, and in light of the findings that reference gene expression is prone to modification by experimental systems, it seems unlikely that under a range of varied experimental conditions the expression of these generic genes will be infinitely stable in this tissue.

Therefore, the aim of this experimental chapter was to identify optimal procedures for sample collection, RNA_{total} isolation, quantification and reverse-transcription; prior to identification of a suitable reference gene from a panel of 7 generic genes for assessing relative expression of angiogenesis-associated growth factor mRNA in human dental pulps isolated from premolar teeth treated with an orthodontic force for two weeks prior to extraction.

3.2 Aims

1. Compare and identify appropriate protocols for sample storage and RNA preservation.
2. Identify a suitable protocol for isolating RNA_{total} from the human dental pulp by optimising homogenisation techniques and comparing standard extraction methods.
3. Identify a suitable protocol for quantifying RNA isolates by comparing well-established universal methodologies.
4. Attempt to maximise the experimental potential of a single dental pulp for RT-qPCR analysis.
5. Identify a suitable reference gene for normalising gene expression data in our unique experimental model.
6. Identify an appropriate method for normalising gene expression data from RT-qPCR experiments by comparing well-established approaches.

3.3 Materials and Methods

MATERIALS

3.3.1 MG-63 Osteosarcoma Cells

Human MG-63 osteosarcoma cells (Cat. No. CRL-1427™) (ATCC® LGC Promochem, Teddington, UK) were provided by Prof A. E. Grigoriadis, Dental Institute, King's College London. RNA_{total} extracted from the cells was used in the construction of standard/calibration curves for reaction efficiency calculation in RT-qPCR analysis, and for optimisation of experimental protocols where pulp samples were limited.

3.3.2 Human Dental Pulps

Following informed parental and patient consent, human dental pulps were obtained from healthy first and second premolar teeth extracted from volunteers aged between 12 – 16 years. All patients included in the study required extraction of a minimum of 2 contralateral premolar teeth in conjunction with fixed appliance orthodontic treatment. Ethical approval was granted by the Research Ethics Committee at Guy's Hospital and King's College Hospital.

METHODS

3.3.3 Cell Culture

MG-63 osteosarcoma cells were grown under sterile conditions at 37 ° C, 5 % CO₂ in Eagle's Alpha Minimum Essential Medium (MEM) (Cat. No. M8042) supplemented with 1 % L-Glutamine (Cat. No. G6784), 1 % Pen-Strep (Cat. No. G6784) and 10 % heat-inactivated Fetal Calf Serum (FCS) (Cat. No. F9665). Cells at passage 5 and a confluence level of 90 – 100 % were trypsinised (0.25 % trypsin solution (Cat. No.

T4049)) and stored as 1 ml aliquots in 40 % Eagle's Alpha MEM (Cat. No. M8042), 50 % FCS (Cat. No. F9665) and 10 % dimethylsulphoxide (Cat. No. C6295) at – 70 °C. All chemicals were sourced from Sigma-Aldrich, Poole, UK

3.3.4 Orthodontic Force Application and Dental Pulp Isolation

In this experimental chapter, pulps were isolated from 8 'control' teeth for the purpose of optimising experimental protocols for sample storage, RNA isolation, RNA quantification, RNA integrity assessment, reverse-transcription and RT-qPCR assay efficiency.

A further 10 contralateral pairs of 'control' and 'treated' teeth, 2 pulps per patient from 10 patients, were isolated in order to identify a stable reference gene for use in RT-qPCR analysis of mRNA expression.

In patients where orthodontic forces were applied to teeth prior to extraction, the oral cavity was divided along the mid-sagittal plane in order to assign a 'control' sample that would forego orthodontic force application; and a contralateral 'treated' sample which would experience a force in the range of 0.5 – 1 N for the duration of treatment. Fixed appliance straight wire orthodontic brackets (Roth 0.022 inch, 3M Unitek, California, USA) were direct bonded (Transbond, 3M Unitek, California, USA) to the upper and/or lower teeth from second premolar to second premolar, except the assigned 'control' teeth. Orthodontic bands were cemented (Ketac, 3M ESPE, Seefeld, Germany) onto all first permanent molar teeth. Test tooth position was carefully assessed and brackets were bonded in a position (offset if necessary) so that the archwires (0.016 inch nickel-titanium, TP Orthodontics Inc., Leeds, UK) placed gave the required force of 0.5 – 1 N in a mesial and extrusive direction (Derringer *et al.* 1996). Following the required period of two weeks of force application, brackets were removed from the premolar teeth assigned as 'treated' teeth.

In all patients, the ‘control’ and contralateral ‘treated’ premolar teeth were extracted under local anaesthesia, placed in individual tubes containing 20 ml of cold sterile saline solution, and sectioned immediately with a high-speed water-cooled diamond bur through the buccolingual aspect. Dental pulps with a mean mass of ± 0.05 g ($n = 5$) were removed from the pulp chamber with sterile blunt instruments. Isolated pulps were then: (a) individually placed in a sterile 1.5 ml centrifuge tube and snap-frozen in liquid nitrogen before transferring to -70 °C for permanent storage; or (b) immersed separately in 500 μ l RNALater™ (Cat. No. AM7021) (Ambion Inc., Warrington, UK), an RNA preservation solution, kept at 4 °C for 8 hr and transferred to -20 °C for permanent storage.

All orthodontic procedures and dental pulp isolations, following tooth extraction, were performed by Dr K. A. Derringer, Dental Institute, King’s College London at Guy’s Hospital and King’s College Hospital.

3.3.5 Pulp Homogenisation for RNA Isolation

3.3.5.1 Mortar and Pestle

Individual snap-frozen pulp samples were submerged in 15 ml TRI Reagent® (Cat. No. AM9738) (Ambion Inc., Warrington, UK), an RNA stabilisation solution, before being ground to a fine powder in a sterile ceramic mortar containing ± 20 ml liquid nitrogen. The powdered pulp was transferred to a sterile tube containing the RNA stabilising solution: (a) 2 ml TRI Reagent® or (b) 600 μ l Buffer RLT (Cat. No. 74104 or 74106) (RNEasy Mini Kit, Qiagen Ltd., Crawley, UK) supplemented with 6 μ l β -mercaptoethanol (Cat. No. M6250) (Sigma-Aldrich, Poole, UK).

RNA_{total} was subsequently isolated from the ground pulps using one of the techniques outlined in section **3.3.6**

3.3.5.2 *Passing Tissue through a Needle*

A snap-frozen pulp sample was immersed in: (a) 1 ml TRI Reagent® or (b) 600 µl Buffer RLT supplemented with 6 µl β-mercaptoethanol; and placed on ice to thaw. Once thawed the sample was passed repeatedly through a 21 gauge sterile needle (BDH, Dorset, UK) with a 1 ml sterile syringe (BDH, Dorset, UK) until entirely shredded.

RNA_{total} was isolated from the shredded pulps using one of the techniques outlined in section **3.3.6**.

3.3.5.3 *FastPrep®-24 Instrument*

Individual snap-frozen pulp samples were transferred to a 2 ml impact resistant FastRNA® Pro Green tube (Cat. No. 116913050) (MPBiomedicals, London, UK) containing: (a) 600 µl TRI Reagent® or (b) 600 µl Buffer RLT supplemented with 6 µl β-mercaptoethanol. Tubes were sealed and positioned in the FastPrep®-24 Instrument (Cat. No. 116004500) (MPBiomedicals, London, UK) according to manufacturer's instructions. Samples were homogenised at 6 ms⁻¹ for 60 sec. Cell debris and ceramic beads were pelleted by centrifuging homogenates at 9,000 g for 5 min at 4 °C. Supernatants were removed and RNA_{total} was isolated using one of the techniques outlined in section **3.3.6**.

3.3.5.4 *Glass Dounce Tissue Homogeniser*

Individual snap-frozen pulp samples or individual pulp samples stored in RNALater™ (Ambion Inc., Warrington, UK) were ground in a 7 ml glass Dounce tissue homogeniser (Wheaton Science Products from VWR, Poole, UK) containing 600 µl of the RNA stabilisation solution RNA-Bee™ (Cat. No. CS-104B or CS-105B or CS-501B) (AMS Biotechnology, Abingdon, UK) and homogenates were transferred to

sterile centrifuge tubes. RNA_{total} was isolated using one of the techniques outlined in section 3.3.6.

3.3.6 RNA Isolation Techniques

3.3.6.1 Phenol:Chloroform & DNase Treatment

400 µl chloroform (Cat. No. C7559) (Sigma-Aldrich, Poole, UK) was added to sample homogenates before vortexing for 15 sec and centrifuging at 9,000 g for 20 min at 4 °C. Supernatants were collected in fresh tubes and 500 µl isopropanol (Cat. No. W292907) (Sigma-Aldrich, Poole, UK) was added before inverting samples and leaving samples for 10 min at room temperature. Samples were centrifuged at 9,000 g for 15 min at 4 °C and supernatants were discarded. RNA_{total} pellets were re-suspended in 700 µl of 70 % ethanol and centrifuged at 9,000 g for 5 min at 4 °C. Supernatants were discarded and pellets were left to dry until ethanol had completely evaporated. Ethanol-free pellets were re-suspended in 172 µl diethyl pyrocarbonate (DEPC)-treated nuclease-free water (Cat. No. AM9906) (Ambion Inc., Warrington, UK).

20 µl of 10 × RQ1 RNase-free DNase reaction buffer (Cat. No. M198A-C) (Promega UK Ltd., Southampton, UK) and 8 µl RQ1 RNase-free DNase (Cat. No. M6101) (Promega UK Ltd., Southampton, UK) was added to the re-suspended RNA_{total} and samples were incubated at 37 °C for 60 min. 200 µl phenol:chloroform:isoamyl alcohol [25:24:1] (Cat. No. P3803) (Sigma-Aldrich, Poole, UK) was added and samples were vortexed for 15 sec prior to centrifugation at 9,000 g for 5 min at 4 °C. Supernatants were transferred to new tubes and 200 µl chloroform:isoamyl alcohol [24:1] (Cat. No. C0549) (Sigma-Aldrich, Poole, UK) was added; samples were vortexed for 15 sec and centrifuged at 9,000 g for 5 min at 4 °C. Supernatants were measured and transferred to new tubes where 1/10 total sample volume 3 M sodium acetate (pH5.2) (Cat. No. S2889) (Sigma-Aldrich, Poole, UK) and 2 × total sample volume 100 % ethanol was

added. Samples were shaken thoroughly and transferred to – 20 °C for 72 hr. Samples were thawed on ice and centrifuged at 9,000 g for 45 min at 4 °C. Supernatants were discarded and the RNA_{total} pellet re-suspended in 700 µl of 70 % ethanol and centrifuged at 9,000 g for 5 min at 4 °C. Supernatants were discarded; pellets were washed in 700 µl of 70 % ethanol and centrifuged at 9,000 g for 5 min at 4 °C. Supernatants were discarded and the pellet left to air-dry until the ethanol had evaporated completely. Ethanol-free RNA_{total} pellets were re-suspended in 20 µl DEPC-treated water (Cat. No. AM9906) (Ambion Inc., Warrington, UK) and transferred to – 70 °C for permanent storage.

3.3.6.2 RNEasy Mini Kit & DNase Treatment

Pulp sample homogenates were transferred to spin columns of the RNEasy Mini Kit (Cat. No. 74104 or 74106) (Qiagen Ltd., Crawley, UK) and processed according to manufacturer's instructions. RNA_{total} was re-suspended in a final volume of 50 µl.

20 µl of 10 × RQ1 RNase-free DNase reaction buffer (Cat. No. M198A-C) (Promega UK Ltd., Southampton, UK), 8 µl RQ1 RNase-free DNase (Cat. No. M6101) (Promega UK Ltd., Southampton, UK) and 90 µl DEPC-treated water (Cat. No. AM9906) (Ambion Inc., Warrington, UK) was added to the re-suspended RNA_{total} and samples were incubated at 37 °C for 60 min. 200 µl phenol:chloroform:isoamyl alcohol [25:24:1] (Cat. No. P3803) (Sigma-Aldrich, Poole, UK) was added and samples were vortexed for 15 sec prior to centrifugation at 9,000 g for 5 min at 4 °C. Supernatants were transferred to new tubes and 200 µl chloroform:isoamyl alcohol [24:1] (Cat. No. C0549) (Sigma-Aldrich, Poole, UK) was added; samples were vortexed for 15 sec and centrifuged at 9,000 g for 5 min at 4 °C. Supernatants were measured and transferred to new tubes where 1/10 total sample volume 3 M sodium acetate (pH5.2) (Cat. No. S2889) (Sigma-Aldrich, Poole, UK) and 400 µl 100 % ethanol was added. Samples

were shaken thoroughly and transferred to – 20 °C for 72 hr. RNA_{total} was pelleted by centrifugation at 9,000 g for 30 min at 4 °C. Supernatants were discarded; the RNA_{total} pellet re-suspended in 800 µl of 70 % ethanol and centrifuged at 9,000 g for 5 min at 4 °C. Supernatants were discarded and the pellet left to air-dry until the ethanol had evaporated completely. Ethanol-free RNA_{total} pellets were re-suspended in 20 µl DEPC-treated water (Cat. No. AM9906) (Ambion Inc., Warrington, UK) and stored permanently at – 70 °C.

3.3.6.3 *Phenol:Chloroform & RNEasy Mini Kit*

60 µl of chloroform (Cat. No. C7559) (Sigma-Aldrich, Poole, UK) was added to the pulp homogenate and samples centrifuged at 4 °C for 15 min at 9,000 g. Supernatants were collected in fresh tubes and a single volume of 70 % ethanol was added. Samples were inverted repeatedly and transferred to spin columns and RNA_{total} extracted using the RNEasy Mini Kit (Cat. No. 74104 or 74106) (Qiagen Ltd., Crawley, UK) according to manufacturer's instructions. Isolated RNA_{total} was suspended in a final volume of 30 µl nuclease-free water (Cat. No. AM9938) (Ambion Inc., Warrington, UK) and stored at -70 °C.

3.3.6.4 *RNA Isolation from MG-63 Osteosarcoma Cells*

RNA_{total} was isolated from snap-frozen 1 ml aliquots of MG-63 osteosarcoma cells using the 'Qias shredder lysis protocol' (Cat. No. 79654 or 79656) (Qiagen Ltd., Crawley, UK) according to manufacturer's instructions. RNA_{total} was suspended in a final volume of 50 µl nuclease-free water (Cat. No. AM9938) (Ambion Inc., Warrington, UK) and stored permanently at – 70 °C.

3.3.7 RNA Quantification Techniques

3.3.7.1 Spectrophotometry

Isolated RNA_{total} was diluted 1:100 with DEPC-treated nuclease-free water (Cat. No. AM9906) (Ambion Inc., Warrington, UK) and the absorbance at 260 nm (A₂₆₀) and 280 nm (A₂₈₀) was measured in a 1 ml quartz cuvette in a standard spectrophotometer. Tare readings were created with 1 ml DEPC-treated water (Cat. No. AM9906) (Ambion Inc., Warrington, UK).

RNA purity was calculated as the A₂₆₀:A₂₈₀ ratio, which correlates to the ratio of nucleic acids and proteins in the sample; and should lie in the range of 1.0 – 2.0, where 2.0 is the most pure.

The RNA_{total} concentration (ng/μl) was calculated as absorbance at 260 nm (A₂₆₀) multiplied by 40, the RNA specific constant, multiplied by the dilution factor, i.e. RNA_{total}(ng/μl) = A₂₆₀ × 40 × (100/1).

The mass (μg) of RNA_{total} isolated from a single sample was calculated as the concentration of RNA_{total} (ng/μl), multiplied by the total sample volume (μl) divided by 1000, i.e. RNA_{total} Mass (μg) = ((A₂₆₀ × 40 × (100/1)) ng/μl × 20 μl) ÷ 1000.

3.3.7.2 NanoDrop Spectrophotometry

The purity and concentration (ng/μl) of RNA_{total} for each sample was determined by calculating the A₂₆₀:A₂₈₀ and A₂₆₀:A₂₃₀ ratios using the NanoDrop™ 1000 spectrophotometer (Now replaced by Cat. No. ND-2000, NanoDrop™ 2000 spectrophotometer) (ThermoFisher Scientific, Nottingham, UK), according to manufacturer's instructions.

3.3.7.3 Ribogreen® RNA Assay Kit

RNA_{total} was quantified by fluorescence with the Quant-iT™ Ribogreen® RNA Assay Kit (Cat. No. R11490) (Molecular Probes, Invitrogen Ltd., Paisley, UK), according to the manufacturer's protocol.

3.3.8 Assessment of RNA Integrity – Bioanalysis of RNA

1 µl of fluorescently labelled RNA_{total} was analysed electrophoretically with the Agilent 2100 Bioanalyzer (Cat. No. G2939AA) (Agilent Technologies, Stockport, UK) on a 6000 Series II Pico chip (Cat. No. 5067-1513) (Agilent Technologies, Stockport, UK) according to manufacturer's instructions. RNA Integrity Numbers (RIN's) were assigned based on the level of sample degradation and solvent contamination. Previously calculated from the ratio of the 18S and 28S rRNA bands, RNA integrity values are now calculated from the entire electrophoretic trace against a scale of 1 – 10. A RIN of 10 indicating that the sample is pure and a RIN of 1 indicating high level degradation (<http://www.chem.agilent.com/rin/rinsearch.aspx>).

3.3.9 Reverse-Transcription Techniques

3.3.9.1 Moloney Murine Leukaemia Virus Reverse Transcriptase

In addition to reverse transcribing RNA to cDNA, a reverse-transcription control (RT control) was created for each sample by replacing the amount of RNA input into a reaction with 1 µl DEPC-treated water (Cat. No. AM9906) (Ambion Inc., Warrington, UK).

3.3.9.1.a Method A

1 µg DNase treated RNA_{total} and 1 µl random primers (Cat. No. C1181) (Promega UK Ltd., Southampton, UK) were added to a sterile centrifuge tube and made up to a

volume of 14 µl with DEPC-treated water (Cat. No. AM9906) (Ambion Inc., Warrington, UK), before heating for 5 min at 70 °C and transferring to ice for 5 min. 5 µl 5 × Moloney Murine Leukaemia Virus (MMLV) Buffer (Cat. No. M5313) (Promega UK Ltd., Southampton, UK), 1.25 µl deoxyribonucleotide triphosphates (dNTPs) (Cat. No, U1511 or U1515) (Promega UK Ltd., Southampton, UK), 1 µl MMLV reverse transcriptase (MMLV-RT) enzyme (Cat. No. M1701) (Promega UK Ltd., Southampton, UK) and 3.75 µl DEPC-treated water (Cat. No. AM9906) (Ambion Inc., Warrington, UK) were added to the sample. Samples were heated at 55 °C for 55 min and 80 °C for 15 min; centrifuged at 9,000 g for 15 sec and stored permanently at – 20 °C.

3.3.9.1.b **Method B**

0.5 µg DNase treated RNA_{total} and 1 µl random primers (Cat. No. C1181) (Promega UK Ltd., Southampton, UK) were added to a sterile centrifuge tube and made up to a volume of 14 µl with DEPC-treated water (Cat. No. AM9906) (Ambion Inc., Warrington, UK), before heating for 5 min at 70 °C and transferring to ice for 5 min. 5 µl of 5 × MMLV Buffer (Cat. No. M5313) (Promega UK Ltd., Southampton, UK), 1.25 µl dNTPs (Cat. No, U1511 or U1515) (Promega UK Ltd., Southampton, UK), 1 µl MMLV-RT enzyme (Cat. No. M1701) (Promega UK Ltd., Southampton, UK) and 3.75 µl DEPC-treated water (Cat. No. AM9906) (Ambion Inc., Warrington, UK) were added to the sample. Samples were heated at 55 °C for 55 min and 80 °C for 15 min; centrifuged at 9,000 g for 15 sec and stored permanently at – 20 °C.

3.3.9.1.c **Method C**

0.5 µg DNase treated RNA_{total} and 0.5 µl random primers were added to a sterile centrifuge tube and made up to a volume of 7 µl with DEPC-treated water (Cat. No. AM9906) (Ambion Inc., Warrington, UK), before heating for 5 min at 70 °C and

transferring to ice for 5 min. 2.5 µl of 5 × MMLV Buffer (Cat. No. M5313) (Promega UK Ltd., Southampton, UK), 0.625 µl dNTPs (Cat. No. U1511 or U1515) (Promega UK Ltd., Southampton, UK), 0.5 µl MMLV-RT enzyme (Cat. No. M1701) (Promega UK Ltd., Southampton, UK) and 1.875 µl DEPC-treated water (Cat. No. AM9906) (Ambion Inc., Warrington, UK) were added to the sample. Samples were heated at 55 °C for 55 min and 80 °C for 15 min; centrifuged at 9,000 g for 15 sec and stored permanently at – 20 °C.

3.3.9.2 *iScript*

10 ng RNA_{total} was incorporated into a 25 µl one-step RT-qPCR reaction made up from 0.5 µl iScript™ reverse transcriptase for one-step RT-PCR (Cat. No. 172-5038) (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK), 0.75 µl forward primer (10 µM) (Eurofins MWG Operon, London, UK), 0.75 µl reverse primer (10 µM) (Eurofins MWG Operon, London, UK), 12.5 µl 2 × SYBR® Green RT-PCR Reaction Mix (Cat. No. 170-8880) (Bio-Rad Laboratories Ltd., UK) and nuclease-free water (Cat. No. AM9938) (Ambion Inc., Warrington, UK).

Amplifications were performed with an initial reverse-transcription step at 50 °C for 10 min, followed by template denaturation at 95 °C for 5 min and 45 cycles of denaturation at 95 °C for 10 sec; combined primer annealing/extension at 60 °C for 30 sec and data acquisition.

A dissociation protocol was performed from 72 °C – 95 °C at 1 °C increments in order to determine analytical specificity and identify primer-dimer or other spurious binding products.

3.3.9.3 *WT-Ovation™ Pico RNA Amplification*

50 ng RNA_{total} was converted to cDNA using the WT-Ovation™ Pico RNA Amplification System (Now replaced by Cat. No. 3302, Ovation Pico WTA System V2)

(NuGen Technologies, Inc., California, USA) according to the manufacturer's protocol. cDNA was then purified using the DNA Clean and Concentrator 25™ kit (Cat. No. D4033 or D4034) (Zymo Research Corporation, California, USA) according to manufacturer's instructions; and quantified with the NanoDrop™ 1000 spectrophotometer. Samples were stored permanently at – 20 °C.

3.3.10 Selection of Candidate Reference Genes and Angiogenic Growth

Factors for mRNA Expression Analysis

A panel of reference genes was selected from three lists of known human reference genes (Eisenberg and Levanon *et al.* 2003, Hsiao *et al.* 2001, Warrington *et al.* 2000) and supplementary microarray data for gene expression in the human dental pulp (Paakkonen *et al.* 2005). Selected genes were representative of a number of cellular functions including metabolism and cell structure, minimising the occurrence of gene co-regulation (**Table 1**).

Genes thought to play a role in the angiogenic response of the human dental pulp to orthodontic force application were selected from a study linking growth factor release in the pulp to orthodontic treatment (Derringer and Linden 2004) (**Table 2**).

Gene Symbol	Gene Name	Gene Function
ACTB	<i>actin, beta</i>	Cytoskeletal structural protein
GAPDH	<i>glyceraldehyde-3-phosphate dehydrogenase</i>	Dehydrogenase
HPRT1	<i>hypoxanthine phosphoribosyltransferase 1</i>	Purine synthesis in salvage pathway
PPIA	<i>peptidylprolyl isomerase A (cyclophilin A)</i>	Catalysis of cis-trans isomerisation
RPL13A	<i>ribosomal protein L13A</i>	60S subunit component involved in translational elongation
UBC	<i>ubiquitin C</i>	Ubiquitination
YWHAZ	<i>tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein, zeta polypeptide</i>	Signal transduction by binding to phosphorylate serine or threonine residues

Table 1 Candidate reference genes

Gene Symbol	Gene Name	Gene Function
FGF2	<i>fibroblast growth factor 2 (basic)</i>	Heparin binding growth factor (mitogenic and angiogenic)
PDGFA	<i>platelet-derived growth factor alpha polypeptide</i>	Collagen binding growth factor (mitogenic)
TGFB1	<i>transforming growth factor, beta 1</i>	Multifunctional mitogenic growth factor
VEGFA	<i>vascular endothelial growth factor A</i>	Endothelial-specific heparin and heparin sulphate binding growth factor (mitogenic and angiogenic)

Table 2 Candidate angiogenic growth factors

3.3.11 Primer Design

Primers (Eurofins MWG Operon, London, UK) for RT-PCR (**Table 3 – Table 7**) and RT-qPCR (**Table 8 – Table 18**) were designed by inspection of the nucleotide sequence and with the Primer-BLAST tool (Basic Local Alignment Search Tool, National Centre for Biotechnology Information) in collaboration with Dr G. Bluteau, Institute of Oral Biology, University of Zurich; or with Primer Express® Software v 2.0 (Applied Biosystems, Warrington, UK) in collaboration with Dr N. Silver, Dental Institute, King's College London.

In order to increase primer specificity and minimise the occurrence of self-binding, primer-dimer or artefact formation; primers were designed across exon-exon boundaries with similar GC contents, lengths and annealing temperatures.

3.3.11.1 *Primers for RT-PCR of Angiogenic Growth Factors and the Reference*

Gene GAPDH

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
FGF2	NM_002006.4	177	Not Applicable for RT-PCR
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
701 - 722	877 - 853	Forward primer	TGCTAACCGTTACCTGGCTATG
Location of Primer by Exon/Intron		Reverse primer	GATCCAAGTTTATACTGCCAGTTC
Spans part of exon 2 and 3			
Secondary Structure of Amplicon		Splice Variants Targeted	
Not assessed		FGF2 - 001, FGF2 - 002 and FGF2 - 201	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
None listed in NCBI database.		http://www.ncbi.nlm.nih.gov/homologene/1521	

Table 3 Primer sequences for angiogenic growth factor FGF2 for RT-PCR

Primer for FGF2 was designed by Dr N. Silver.

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
PDGFA	NM_033023	202	Not Applicable for RT-PCR
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
1241 - 1260	1442 - 1423	Forward primer	GCAACACGAGCAGTGTCAAG
Location of Primer by Exon/Intron		Reverse primer	GCTCATCCTCACCTCACATC
Spans part of exon 4 and 5			
Secondary Structure of Amplicon		Splice Variants Targeted	
Not assessed		PDGFA - 001, PDGFA - 002 and PDGFA - 005	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
None listed in NCBI database.		http://www.ncbi.nlm.nih.gov/homologene/32055	

Table 4 Primer sequences for angiogenic growth factor PDGFA for RT-PCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
TGFB1	NM_000660	319	Not Applicable for RT-PCR
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3') Forward primer CTATCGACATGGAGCTGGTGA Reverse primer CGGAGCTCTGATGTGTTGAAG	
993 - 1013	1311 - 1291		
Location of Primer by Exon/Intron Spans part of exon 1 and 2		Splice Variants Targeted TGFB1 - 001	
Secondary Structure of Amplicon Not assessed		Homologs listed at: http://www.ncbi.nlm.nih.gov/homologene/540	
Pseudogenes or retropseudogenes listed at: None listed in NCBI database.			

Table 5 Primer sequences for angiogenic growth factor TGFB1 for RT-PCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
VEGFA	BC065522	593	Not Applicable for RT-PCR
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3') Forward primer CTTGCCTTGCTGCTCTACC Reverse primer AAGTGCTCTGCGCAGAGTC	
499 - 517	1091 - 1073		
Location of Primer by Exon/Intron No data given by Primer-BLAST		Splice Variants Targeted VEGFA - 001, VEGFA - 002, VEGFA - 003, VEGFA - 018, VEGFA - 019, VEGFA - 020, VEGFA - 201, VEGFA - 202, VEGFA - 203 and VEGFA - 204	
Secondary Structure of Amplicon Not assessed		Homologs listed at: http://www.ncbi.nlm.nih.gov/homologene/2534	
Pseudogenes or retropseudogenes listed at: None listed in NCBI database.			

Table 6 Primer sequences for angiogenic growth factor VEGFA for RT-PCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
GAPDH	NM_002046	433	Not Applicable for RT-PCR
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
729 - 748	1171 - 1152	Forward primer	ATCACTGCCACCCAGAAGAC
Location of Primer by Exon/Intron		Reverse primer	ATGAGGTCCACCACCCTGTT
Spans part of exon 8 and 9		Splice Variants Targeted	
Secondary Structure of Amplicon		GAPDH - 001, GAPDH - 002, GAPDH - 003, GAPDH - 004, GAPDH - 005, GAPDH - 006, GAPDH - 007, GAPDH - 008 and GAPDH - 201	
Not assessed		Homologs listed at:	
Pseudogenes or retropseudogenes listed at:		http://www.ncbi.nlm.nih.gov/homologene/107053	
http://www.ncbi.nlm.nih.gov/gene/?Term=related_functional_gene_2597[group]			

Table 7 Primer sequences for reference gene GAPDH for RT-PCR

Primer for GAPDH was designed by Dr G. Bluteau.

3.3.11.2 Primers for RT-qPCR of Angiogenic Growth Factors

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
FGF2	NM_002006.4	117	1.00
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
601 - 617	717 - 695	Forward primer	CCGACGGCCGAGTTGAC
Location of Primer by Exon/Intron		Reverse primer	CCAGGTAACGGTTAGCACACT
Spans part of exon 1 and 2		Splice Variants Targeted	
Secondary Structure of Amplicon		FGF2 - 001, FGF2 - 002 and FGF2 -201	
Not assessed		Homologs listed at:	
Pseudogenes or retropseudogenes listed at:		http://www.ncbi.nlm.nih.gov/homologene/1521	
None listed in NCBI database			

Table 8 Primer sequences for angiogenic growth factor FGF2 for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
PDGFA	NM_033023	96	1.00
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
1065 - 1079	1160 - 1139	Forward primer	GCCCGAGAAGCGGCC
Location of Primer by Exon/Intron		Reverse primer	GGAATCTCGTAAATGACCGTCC
Spans part of exon 3 and 4			
Secondary Structure of Amplicon		Splice Variants Targeted	
Not assessed		PDGFA - 001, PDGFA - 002, PDGFA - 003 and PDGFA - 005	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
None listed in NCBI database		http://www.ncbi.nlm.nih.gov/homologene/32055	

Table 9 Primer sequences for angiogenic growth factor PDGFA for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
TGFB1	NM_000660	79	1.00
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
1358 - 1381	1436 - 1416	Forward primer	CTGCTGAGGCTCAAGTTAAAGTG
Location of Primer by Exon/Intron		Reverse primer	TGAGGTATCGCCAGGAATTGT
Spans part of exon 2 and 3			
Secondary Structure of Amplicon		Splice Variants Targeted	
Not assessed		TGFB1 - 001 and TGFB1 - 002	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
None listed in NCBI database		http://www.ncbi.nlm.nih.gov/homologene/540	

Table 10 Primer sequences for angiogenic growth factor TGFB1 for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
VEGFA	BC065522	101	0.91
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
749 - 767	849 - 826	Forward primer	TGCCCACTGAGGAGTCCAA
Location of Primer by Exon/Intron		Reverse primer	ACATTTGTTGTGCTGTAGGAAGCT
No data given by Primer-BLAST			
Secondary Structure of Amplicon		Splice Variants Targeted	
Not assessed		VEGFA - 001, VEGFA - 002, VEGFA - 003, VEGFA - 004, VEGFA - 005, VEGFA - 007, VEGFA - 009, VEGFA - 011, VEGFA - 012, VEGFA - 013, VEGFA - 014, VEGFA - 015, VEGFA - 016, VEGFA - 017, VEGFA - 018, VEGFA - 019, VEGFA - 020, VEGFA - 021, VEGFA - 022, VEGFA - 023, VEGFA - 024, VEGFA - 025, VEGFA - 201, VEGFA - 202, VEGFA - 203 and VEGFA - 204	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
None listed in NCBI database		http://www.ncbi.nlm.nih.gov/homologene/2534	

Table 11 Primer sequences for angiogenic growth factor VEGFA for RT-qPCR

3.3.11.3 *Primers for RT-qPCR of Reference Genes*

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
ACTB	NM_001101.3	203	0.58
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
271 - 190	473 - 454	Forward primer	GGCATCCTCACCCTGAAGTA
Location of Primer by Exon/Intron		Reverse primer	GGGGTGTGAAGGTCTCAA
Spans part of exon 3 and 4			
Secondary Structure of Amplicon		Splice Variants Targeted	
Not assessed		ACTB - 001, ACTB - 002, ACTB - 003, ACTB - 004, ACTB - 005, ACTB - 007, ACTB - 008 and ACTB - 011	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
http://www.ncbi.nlm.nih.gov/gene/?Term=related_functional_gene_60[group]		http://www.ncbi.nlm.nih.gov/homologene/110648	

Table 12 Primer sequences for candidate reference gene ACTB for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
GAPDH	NM_002046.5	112	0.86
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
171 - 190	282 - 254	Forward primer	CCACATCGCTCAGACACCAT
Location of Primer by Exon/Intron		Reverse primer	CAACAATATCCACTTTACCAGAGTAAAA
Spans part of exon 2 and 3			
Secondary Structure of Amplicon		Splice Variants Targeted	
Not assessed		GAPDH - 001, GAPDH - 002, GAPDH - 003, GAPDH - 004, GAPDH - 005, GAPDH - 006, GAPDH - 007 and GAPDH - 011	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
http://www.ncbi.nlm.nih.gov/gene/?Term=related_functional_gene_2597[group		http://www.ncbi.nlm.nih.gov/homologene/107053	

Table 13 Primer sequences for candidate reference gene GAPDH for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
HPRT1	NM_000194.2	139	0.82
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
267 - 291	405 - 384	Forward primer	AGGGTGTTTATTCCTCATGGACTAA
Location of Primer by Exon/Intron		Reverse primer	CCAGCAGGTCAGCAAAGAATTT
Spans part of exon 2 and 3			
Secondary Structure of Amplicon		Splice Variants Targeted	
Not assessed		HPRT1 - 001, HPRT1 - 002 and HPRT1 - 003	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
http://www.ncbi.nlm.nih.gov/gene/?Term=related_functional_gene_3251[group		http://www.ncbi.nlm.nih.gov/homologene/56590	

Table 14 Primer sequences for candidate reference gene HPRT1 for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
PPIA	NM_021130.4	73	0.42
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
141 - 157	213 - 192	Forward primer	GGCCGCGTCTCCTTTGA
Location of Primer by Exon/Intron		Reverse primer	CAGTGCTCAGAGCAGAAAATT
Spans part of exon 2 and 3			
Secondary Structure of Amplicon		Splice Variants Targeted	
Not assessed		PPIA - 001, PPIA - 002, PPIA - 003, PPIA - 005, PPIA - 006, PPIA - 007, PPIA - 009 and PPIA - 201	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
http://www.ncbi.nlm.nih.gov/gene/?Term=related_functional_gene_5478[group		http://www.ncbi.nlm.nih.gov/homologene/134504	

Table 15 Primer sequences for candidate reference gene PPIA for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
RPL13A	NM_012423.3	126	0.53
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
541 - 563	666 - 642	Forward primer	CCTGGAGGAGAAGAGGAAAGAGA
Location of Primer by Exon/Intron		Reverse primer	TTGAGGACCTCTGTGTATTTGTCAA
Spans part of exon 7 and 8			
Secondary Structure of Amplicon		Splice Variants Targeted	
Not assessed		RPL13A - 001, RPL13A - 002, RPL13A - 003, RPL13A - 004, RPL13A - 006, RPL13A - 008, RPL13A - 009, RPL13A - 012, RPL13A - 013 and RPL13A - 201	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
http://www.ncbi.nlm.nih.gov/gene/?Term=related_functional_gene_23521[group		http://www.ncbi.nlm.nih.gov/homologene/111053	

Table 16 Primer sequences for candidate reference gene RPL13A for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
UBC	NM_021009.6	132	0.64
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
2350 - 2372	2481 - 2457	Forward primer	TCGAGAATGTCAAGGCCAAAGATC
Location of Primer by Exon/Intron		Reverse primer	GAGTGGACTCTTTCTGGATGTTGTA
Spans part of exon 2			
Secondary Structure of Amplicon		Splice Variants Targeted	
Not assessed		UBC - 001, UBC - 003, UBC - 007 and UBC - 201	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
None listed on NCBI website		http://www.ncbi.nlm.nih.gov/homologene/128418	

Table 17 Primer sequences for candidate reference gene UBC for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
YWHAZ	NM_145690.2	196	0.71
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
519 - 540	714 - 692	Forward primer	AAGTTCTTGATCCCCAATGCTT
Location of Primer by Exon/Intron		Reverse primer	GTCTGATAGGATGTGTTGGTTGC
Spans part of exon 3 and 4			
Secondary Structure of Amplicon		Splice Variants Targeted	
Not assessed		YWHAZ - 001, YWHAZ - 002, YWHAZ - 003, YWHAZ - 004, YWHAZ - 008, YWHAZ - 009, YWHAZ - 010, YWHAZ - 011, YWHAZ - 012, YWHAZ - 013, YWHAZ - 015, YWHAZ - 016, YWHAZ - 017, YWHAZ - 022, YWHAZ - 201 and YWHAZ - 202	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
http://www.ncbi.nlm.nih.gov/gene/?Term=related_functional_gene_7534[group		http://www.ncbi.nlm.nih.gov/homologene/56528	

Table 18 Primer sequences for candidate reference gene YWHAZ for RT-qPCR

3.3.12 Reverse-Transcription PCR (RT-PCR)

All materials in this experiment were sourced from Promega UK Ltd., Southampton, UK with the exception of DEPC-treated water (Cat. No. AM9906) and the PCR oil

(Cat. No. M8662-5VL), which was sourced from Ambion Inc., Warrington, UK and Sigma-Aldrich, Poole, UK respectively.

50 µl reactions were made up from 10 µl GoTaq™ Buffer (Cat. No. M8305), 3 µl MgCl²⁺ (1.5mM) (Cat. No. A3513), 1 µl dNTPs (Cat. No. U1511 or U1515), 1 µl forward primer (0.1 µg/µl) (Eurofins MWG Operon, London, UK), 1 µl reverse primer (0.1 µg/µl) (Eurofins MWG Operon, London, UK), 0.5 µl Taq polymerase enzyme (Cat. No. M891A), 32.5 µl DEPC-treated water (Cat. No. AM9906) and: (a) 1 µl cDNA, (b) 1 µl DEPC-treated water (Cat. No. AM9906) or (c) 1 µl RT control (section **3.3.9.1**). 50 µl PCR oil (Cat. No. M8662-5VL) was then added to the top of each reaction to ensure even heat distribution in a thermal cycler with no top heat block.

The general procedure for RT-PCR was: Initial denaturation at 95 °C for 2 min; 35 cycles of 94 °C for 40 sec (denaturation), 60 °C for 40 sec (annealing), 72 °C for 30 sec (extension); followed by a final extension of 5 min at 72 °C.

3.3.13 Reverse-Transcription Quantitative Real-Time PCR (RT-qPCR)

Analysis of mRNA Expression

RT-qPCR was performed using the Corbett Rotor-Gene™ 6000 (Corbett Life Sciences, Qiagen Ltd., Crawley, UK) on 25 µl one-step reverse-transcription reactions using iScript™ reverse transcriptase with SYBR® Green (Cat. No. 172-5038) (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK).

Reactions were set up manually in 0.1 ml strip tubes (Cat. No. 981103) (Qiagen Ltd., Crawley, UK) and 10 ng RNA_{total} was added to 0.5 µl iScript™ reverse transcriptase for one-step RT-PCR (Cat. No. 172-5038) (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK), 0.75 µl forward primer (10 µM) (Eurofins MWG Operon, London, UK), 0.75 µl reverse primer (10 µM) (Eurofins MWG Operon, London, UK), 12.5 µl 2 × SYBR®

Green RT-PCR Reaction Mix (Cat. No. 170-8880) (Bio-Rad Laboratories Ltd., UK) and nuclease-free water (Cat. No. AM9938) (Ambion Inc., Warrington, UK).

Amplifications were performed with an initial reverse-transcription step at 50 °C for 10 min, followed by template denaturation at 95 °C for 5 min and 45 cycles of denaturation at 95 °C for 10 sec; combined primer annealing/extension at 60 °C for 30 sec and data acquisition. A dissociation protocol was performed from 72 °C – 95 °C at 1 °C increments in order to determine analytical specificity and identify primer-dimer or other spurious binding products.

To verify RT-qPCR amplification product size, a dissociation protocol was omitted for each of the genes of interest (GOIs) and products were separated by gel electrophoresis. Standard curves were constructed from a 1:10 dilution series of MG-63 cDNA (20 ng/reaction – 0.0002 ng/reaction) in order to calculate RT-qPCR reaction efficiencies.

Details of primers are given in **Table 8 – Table 18**.

3.3.14 Gel Electrophoresis

RT-PCR products and RT-qPCR products from the iScript protocol were separated according to molecular weight by gel electrophoresis.

20 µl RT-PCR product and 2 µl gel-loading buffer (Cat. No. G2526-5ML) (Sigma-Aldrich, Poole, UK) was loaded onto a 2 % agarose (Cat. No. BIO-41025) (Bioline Ltd., London, UK) gel made with 0.5 × TrisBorateEDTA (TBE) buffer (Cat. No. T4415) (Sigma-Aldrich, Poole, UK) and 2 µl ethidium bromide (Cat. No. E1385) (Sigma-Aldrich, Poole, UK). Samples were run in 0.5 × TBE buffer for 40 min at 100 V alongside a 100 bp DNA ladder (Cat. No. G2101) (Promega UK Ltd., Southampton, UK) and gels were photographed using a UV transilluminator linked to a printer.

5 μ l RT-qPCR product and 0.83 μ l 6 \times blue/orange loading dye (Cat. No. G1881) (Promega UK Ltd., Southampton, UK) were loaded onto a 2 % agarose gel made with 0.5 \times TBE buffer and 2 μ l GelRed™ Nucleic Acid Gel Stain (Cat. No. 41003) (Biotium Inc., Cambridge BioScience Ltd., Cambridge, UK). Samples were run in 0.5 \times TBE buffer for 15 min at 100 V alongside a 100 bp ladder and gels were photographed using a UV transilluminator linked to a printer.

3.3.15 Statistical Analysis of RT-qPCR Data

3.3.15.1 Reference Gene Expression Stability Analysis

Raw RT-qPCR data was analysed with Corbett Rotor-Gene™ 6000 Application Software, version 1.7 (Build 87) and expressed as quantification cycle (C_q) values. Gene stability was initially assessed by analysing gene expression distribution and normalising to RNA_{total} by performing statistical analysis with the paired Student's t-Test. C_q values were inserted into the Microsoft Excel-based tool, *BestKeeper*© version 1 (Pfaffl *et al.* 2004, <http://normalisation.gene-quantification.info/>) and GenEx software version 4.3.5 (MultiD Analyses AB, Göteborg, Sweden and TATAA Biocenter, Göteborg, Sweden) (<http://normalisation.gene-quantification.info/>) which includes, among other useful BioInformatic tools, the geNorm (Vandesompele *et al.* 2002) and NormFinder (Andersen *et al.* 2004) algorithms. Statistical significance was given at $p \leq 0.05$

3.3.15.2 Gene Expression Analysis for Angiogenic Growth Factors

Relative mRNA expression of angiogenic growth factors (**Table 2**) was compared between 'control' and contralateral 'treated' pulps using the Delta C_t method (Schmittgen and Livak 2008) which compares the fold-change in gene expression by analysing each sample as an individual, isolated data point; and the Delta-Delta C_t

(Livak and Schmittgen 2001, Schmittgen and Livak 2008) method which compares the fold-change in gene expression by pairing the contralateral ‘treated’ and ‘control’ pulps together. C_q values were also input into the REST-2009© software package (developed by Prof. M. Pfaffl, Technical University Munich, Germany and Qiagen Ltd., Crawley, UK; <http://www.gene-quantification.com/rest-2009.html>) for relative expression analysis with efficiency correction. Statistical significance was given at $p \leq 0.05$.

3.4 Results

3.4.1 Sample Storage and RNA Preservation

Student's t-Test assuming unequal variances ($n = 5$) showed that there was no statistically significant difference ($p > 0.05$) in the amount (ng) of RNA_{total} isolated from human dental pulp samples preserved by snap-freezing, mean (\pm SEM) of 2,816.98 \pm 1,208.81 ng, when compared with storage in an RNA preservative, mean (\pm SEM) of 2,126.56 \pm 833.42 ng. Values were obtained with the NanoDrop™ 1000 spectrophotometer (section 3.3.7.2).

Both preservation methods were assumed to result in similar yields (ng) of RNA_{total} ($p = 0.66$), and so RNeasy™ was utilised for sample collection for mRNA expression analysis due to its safety advantages in the dental clinical environment and in transportation between the dental clinics and the laboratory; while snap-freezing was utilised for protocol optimisation as samples had already been collected using this technique.

3.4.2 RNA Isolation, Yield and Sample Purity

3.4.2.1 Optimisation of Tissue Homogenisation and RNA Isolation Protocols

RNA_{total} isolated from snap-frozen dental pulp tissue, homogenised with a mortar and pestle ($n = 2$, section 3.3.5.1) and isolated by: (a) Phenol:Chloroform & DNase Treatment ($n = 1$, section 3.3.6.1) or (b) RNeasy Mini Kit & DNase Treatment ($n = 1$, section 3.3.6.2); or homogenised by passing the sample through a needle in RNA stabilisation solution ($n = 2$, section 3.3.5.2) and isolated by: (a) Phenol:Chloroform & DNase Treatment ($n = 1$, section 3.3.6.1) (b) RNeasy Mini Kit & DNase Treatment ($n = 1$, section 3.3.6.2) yielded RNA_{total} amounts of ≥ 0.5 μ g per pulp when quantified

by standard spectrophotometry (section **3.3.7.1**). Spectrophotometry indicated that there was little difference in sample integrity (A_{260} : A_{280} ratio).

Snap-frozen samples homogenised: (a) with a mortar and pestle ($n = 1$, section **3.3.5.1**) and suspended in supplemented Buffer RLT (section **3.3.6.2**); (b) by passing the sample through a needle ($n = 1$, section **3.3.5.2**) in supplemented Buffer RLT (section **3.3.6.2**); (c) pulverised in supplemented Buffer RLT in the FastPrep®-24 instrument ($n = 1$, sections **3.3.5.3** and **3.3.6.2**); or (d) in a glass homogeniser in combination with the modified phenol:chloroform RNA isolation protocol ($n = 1$, section **3.3.5.4** and **3.3.6.3**) were processed according to manufacturer's instructions for RNA isolation with the RNEasy Mini Kit. RNA_{total} was quantified by NanoDrop Spectrophotometry (section **3.3.7.2**) and sample integrity was assessed with the Agilent 2100 Bioanalyzer (section **3.3.8**) (**Table 19**).

Tissue Homogenisation	Mortar & Pestle	Needle	Fast-Prep®-24	Glass Homogeniser
RIN Value	2.90	5.30	Not Calculated	5.40
Electrophoretogram				

Table 19 Bioanalyzer RIN values and gel electrophoretograms

Electrophoretic traces (**Figure 2 – Figure 5**) constructed by the 2100 Expert Software, from the gel electrophoretograms shown in **Table 19**, illustrate the level of sample degradation and the defined peaks assigned to the 18S and 28S ribosomal RNA subunits. In samples homogenised by mortar and pestle in liquid nitrogen (**Figure 2**) or passed through a needle (**Figure 3**), the peaks representative of the ribosomal subunits were small and indistinguishable; samples were shown to have high levels of degradation and despite being treated with DNase, contained large concentrations of genomic DNA indicated as a continuous peak between the 18S and 28S ribosomal RNA subunits. When the pulp was homogenised by the FastPrep®-24 machine, peaks representative of the ribosomal subunits were well defined and the remainder of the sample showed little degradation (**Figure 4**). When homogenised with the glass Dounce tissue homogeniser and isolated by the modified phenol:chloroform protocol, subunit peaks were well defined, however the remainder of the sample showed a low level of degradation (**Figure 5**).

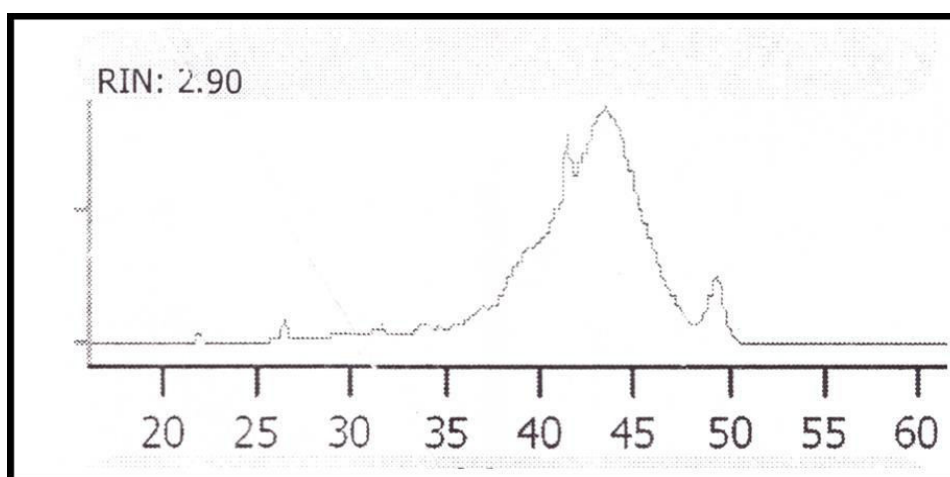


Figure 2 Bioanalysis of RNA from pulp homogenised by mortar and pestle

Electrophoretic trace illustrating sample degradation and RNA integrity. Sample shows a large undefined 18S ribosomal peak at 42 followed by a wide peak of genomic DNA (43 – 46) and a small 28S ribosomal RNA peak at 49.

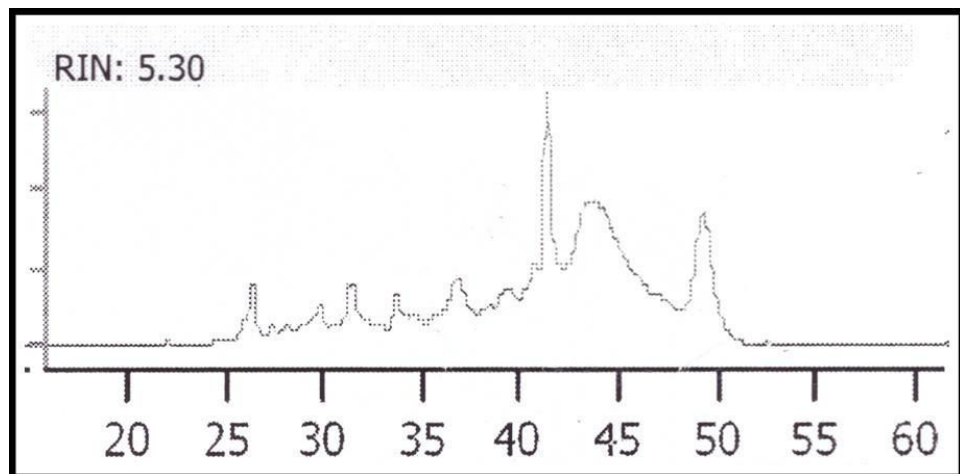


Figure 3 Bioanalysis of RNA from pulp homogenised by passing pulp through a needle

Electrophoretic trace illustrating sample degradation and RNA integrity. Sample shows a large undefined 18S ribosomal peak at 42 followed by a peak of genomic DNA (43 – 46) and a defined 28S ribosomal RNA peak at 49.

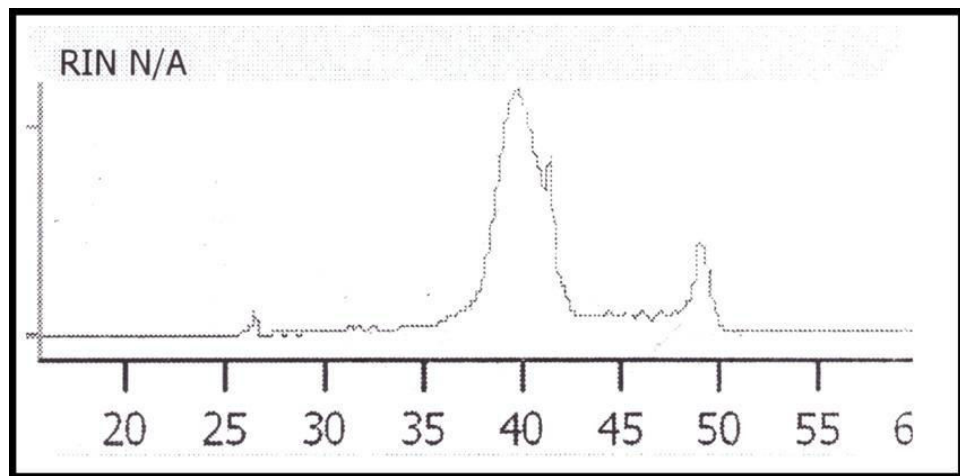


Figure 4 Bioanalysis of RNA from pulp homogenised by FastPrep®-24

Electrophoretic trace illustrating sample degradation and RNA integrity. Sample shows a large defined 18S ribosomal peak at 42 followed a small 28S ribosomal RNA peak at 49. This sample showed no genomic DNA contamination.

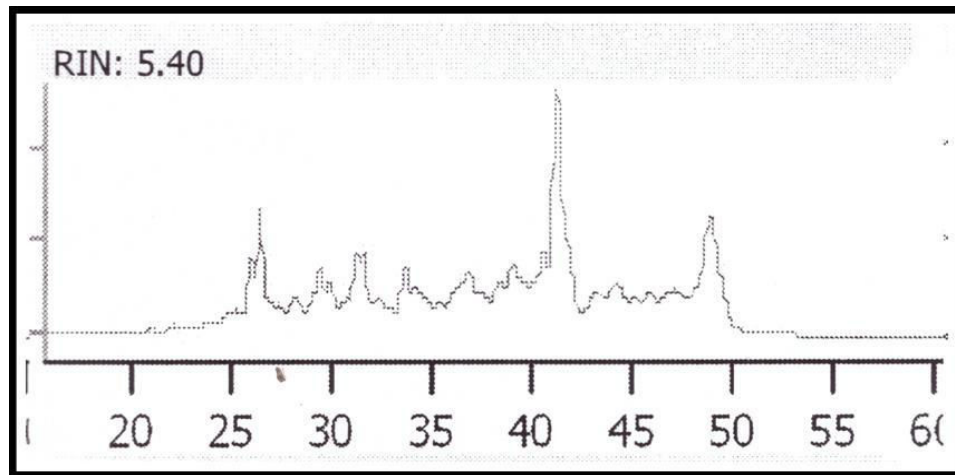


Figure 5 Bioanalysis of RNA from pulp homogenised in a glass Dounce tissue homogeniser

Electrophoretic trace illustrating RNA degradation and sample integrity. Sample shows a large defined 18S ribosomal peak at 42 followed by minimal genomic DNA (43 – 46) and a large 28S ribosomal RNA peak at 49.

RNA_{total} isolated by the RNEasy Mini Kit subsequent to homogenisation in the FastPrep®-24 instrument yielded the highest concentration of RNA_{total} (2.34 ng/μl) with the lowest level of degradation from all tested combinations although no RNA Integrity Number (RIN) could be assigned (**Figure 4**). RNA_{total} isolated by the modified phenol:chloroform extraction method subsequent to homogenisation in the glass Dounce tissue homogeniser yielded a marginally lower concentration of RNA_{total} (1.44 ng/μl) with similarly strong peaks to those of the FastPrep®-24 instrument and a RIN of 5.40. As the glass Dounce tissue homogenisers were readily available at no additional cost to the laboratory, this combination of homogenisation and RNA isolation was used from this point forward.

3.4.2.2 Optimisation of RNA Quantification Protocols

Standard spectrophotometry was discounted as an accurate method for RNA quantification as yields of ≤ 0.5 μg per pulp (n = 4) were below the limit of detection (LOD) for this assay (Bustin 2000, Fleige and Pfaffl 2006).

2 Weeks Orthodontic Force Application							
Control Pulp	NanoDrop Spectrophotometer				Ribogreen Assay		
	A260:A280	A260:A230	RNA _{total} Yield (ng/μl)	RNA _{total} Yield per Pulp (ng)	RNA _{total} Yield (ng/μl)	RNA _{total} Yield per Pulp (ng)	
1	2.01	1.44	94.65	2839.40	25.56	766.80	
2	1.87	0.81	62.20	1866.00	21.71	651.30	
3	1.96	0.75	66.36	1990.70	29.13	873.90	
4	2.00	1.92	296.33	8890.00	34.52	1035.60	
5	1.91	0.34	36.24	1087.30	15.49	464.70	
6	1.96	1.48	81.51	2445.20	29.67	890.10	
7	1.71	0.81	44.49	1334.80	23.56	706.80	
8	1.91	1.08	86.15	2584.50	26.80	804.00	
9	1.92	0.71	52.10	1562.90	26.44	793.20	
10	1.96	1.34	65.18	1955.30	27.80	834.00	
Mean ± SEM	1.92 ± 0.03	1.07 ± 0.15	88.52 ± 23.81	2655.61 ± 714.18	26.07 ± 1.62	782.40 ± 48.47	

Treated Pulp	NanoDrop Spectrophotometer				Ribogreen Assay		
	A260:A280	A260:A230	RNA _{total} Yield (ng/μl)	RNA _{total} Yield per Pulp (ng)	RNA _{total} Yield (ng/μl)	RNA _{total} Yield per Pulp (ng)	
1	2.11	1.77	96.25	2887.60	31.44	943.20	
2	1.92	0.64	50.62	1518.60	24.65	739.50	
3	1.99	1.34	129.19	3875.70	33.33	999.90	
4	2.01	2.03	358.08	10742.40	32.58	977.40	
5	1.92	0.82	51.71	1551.40	16.82	504.60	
6	1.94	1.04	100.08	3002.30	31.33	939.90	
7	1.89	1.37	56.40	1692.10	27.71	831.30	
8	1.99	1.73	104.89	3146.80	28.41	852.30	
9	1.93	1.41	45.04	1351.30	25.78	773.40	
10	1.92	0.87	46.43	1392.80	24.96	748.80	
Mean ± SEM	1.96 ± 0.02	1.30 ± 0.14	103.87 ± 29.80	3116.10 ± 893.90	27.70 ± 1.57	831.03 ± 47.12	

Table 20 RNA_{total} yields from human dental pulps when quantified by NanoDrop spectrophotometry and fluorescence (Ribogreen assay)

RNA yields ranged from 1,087.30 – 10,742.40 ng per sample with a mean (\pm SEM) yield of $2,885.86 \pm 559.33$ ng RNA_{total} per pulp sample (n = 20, **Table 20**) when quantified with the NanoDrop™ 1000 spectrophotometer. However, this mean yield was determined to be 3.5-times lower when the same samples were quantified by fluorescence with the Ribogreen® RNA Assay Kit (464.70 – 1,035.60 ng per sample with a mean (\pm SEM) yield of 806.54 ± 33.37 ng RNA_{total} per pulp sample (n = 20, **Table 20**). By paired Student's t-Test it was identified that there was a statistically significant difference between the two quantification techniques ($p < 0.0005$), and a Pearson's correlation coefficient of 0.559 was assigned, indicating that the methodologies are not tightly correlated.

The mean (\pm SEM) A₂₆₀:A₂₈₀ (nucleic acid to protein) ratio was 1.94 ± 0.02 (ranging from 1.71 – 2.11) (n = 20, **Table 20**). The mean (\pm SEM) A₂₆₀:A₂₃₀ (nucleic acid to

organic compound contamination) ratio was 1.19 ± 0.10 (range from 0.34 – 2.03) (n = 20, **Table 20**).

NanoDrop Spectrometry was used in conjunction with Ribogreen® quantification for the duration of the study as the fluorescence assay, despite being more appropriate for our experimental model, could not determine the sample quality by assessing the $A_{260}:A_{280}$ or $A_{260}:A_{230}$ ratios. Samples were found to be within the acceptable range of good quality RNA_{total} as per Sambrook *et al* (1989) which states that an $A_{260}:A_{280}$ ratio greater than 1.8 is acceptable.

3.4.3 Reverse-Transcription for RT-PCR and RT-qPCR

3.4.3.1 Optimisation of Reverse-Transcription Protocols for RT-PCR

RNA_{total} extracted from 1 µl MG-63 osteosarcoma cell aliquots (section **3.3.6.4**) and quantified by standard spectrophotometry (section **3.3.7.1**) was reverse transcribed by the three Moloney Murine Leukaemia Virus Reverse Transcriptase methods outlined in section **3.3.9.1.a – 3.3.9.1.c**. RT-PCR was performed with primers for GAPDH (**Table 7**) and RT-PCR products were separated by gel electrophoresis (section **3.3.14**). The gel electrophoretogram (**Figure 6**) illustrates that by halving the original input amount of RNA_{total} to 0.5 µg as per method **3.3.9.1.b** (result shown in Lane 5, **Figure 6**), the cDNA yield from a single reverse-transcription reaction is relative to that obtained by reverse transcribing a full 1 µg RNA_{total} as per method **3.3.9.1.a** (result shown in Lane 2, **Figure 6**). By halving both the RNA_{total} input and the reagent volumes as per method **3.3.9.1.c**, the cDNA yield is reduced by more than half (result shown in Lane 8, **Figure 6**). For the purpose of RT-PCR, method **3.3.9.1.b** was used for reverse transcribing RNA_{total} from this point onward as it maximised the number of reactions that could be performed with a single pulp. These observations are purely qualitative.

2 Weeks Orthodontic Force Application					
	Ribogreen Assay	iScript one-step RT-qPCR Kit	WT-Ovation™ Pico RNA Amplification System		
Control Pulp	RNA _{total} Yield per Pulp (ng)	Number of Reactions per Pulp (10 ng/reaction)	Number of Reactions per Pulp (50 ng/reaction)	cDNA Yield from Single Run (ng)	Potential cDNA Yield per Pulp (ng)
1	766.80	76.68	15.34	6300.80	96629.07
2	651.30	65.13	13.03	9038.40	117734.20
3	873.90	87.39	17.48	6452.40	112775.05
4	1035.60	103.56	20.71	8083.90	167433.74
5	464.70	46.47	9.29	7664.20	71231.07
6	890.10	89.01	17.80	7894.10	140530.77
7	706.80	70.68	14.14	10458.90	147847.01
8	804.00	80.40	16.08	8739.90	140537.59
9	793.20	79.32	15.86	8980.80	142471.41
10	834.00	83.40	16.68	4529.90	75558.73
Mean ± SEM	782.04 ± 48.47	78.20 ± 4.85	15.64 ± 0.97	7814.33 ± 534.83	121274.86 ± 10173.17
	Ribogreen Assay	iScript one-step RT-qPCR Kit	WT-Ovation™ Pico RNA Amplification System		
Treated Pulp	RNA _{total} Yield per Pulp (ng)	Number of Reactions per Pulp (10 ng/reaction)	Number of Reactions per Pulp (50 ng/reaction)	cDNA Yield from Single Run (ng)	Potential cDNA Yield per Pulp (ng)
1	943.20	94.32	18.86	7259.60	136945.09
2	739.50	73.95	14.79	8180.30	120986.64
3	999.90	99.99	20.00	9588.00	191740.82
4	977.40	97.74	19.55	7866.50	153774.34
5	504.60	50.46	10.09	8623.10	87024.33
6	939.90	93.99	18.80	9420.80	177092.20
7	831.30	83.13	16.63	7946.10	132111.86
8	852.30	85.23	17.05	10304.70	175653.92
9	773.40	77.34	15.47	10029.90	155142.49
10	748.80	74.88	14.98	2229.20	33384.50
Mean ± SEM	831.03 ± 55.56	83.10 ± 5.56	16.62 ± 1.11	8144.82 ± 573.16	136385.62 ± 11853.22
	Ribogreen Assay	iScript one-step RT-qPCR Kit	WT-Ovation™ Pico RNA Amplification System		
All Samples	RNA _{total} Yield per Pulp (ng)	Number of Reactions per Pulp (10 ng/reaction)	Number of Reactions per Pulp (50 ng/reaction)	cDNA Yield from Single Run (ng)	Potential cDNA Yield per Pulp (ng)
Mean ± SEM	806.54 ± 33.37	80.65 ± 3.34	16.13 ± 0.67	7979.58 ± 442.32	128830.24 ± 8984.63
	iScript one-step RT-qPCR Kit	WT-Ovation™ Pico RNA Amplification System			
All Samples	Potential Number of Reactions per Pulp In Technical Duplicate (10 ng RNA _{total} /reaction)	Potential Number of Reactions in Technical Duplicate from a Single Run (10 ng cDNA/reaction)	Potential Number of Reactions in Technical Duplicate per Pulp (10 ng cDNA/reaction)		
Mean ± SEM	40.33 ± 1.67	398.96 ± 22.12	6441.51 ± 449.23		

Table 21 Comparison between the iScript one-step RT-qPCR kit and the WT-Ovation™ Pico RNA Amplification System

Using the known mean RNA_{total} yield per pulp sample (n = 20) the experimental potential of a single pulp was determined for RT-qPCR analysis. The iScript one-step RT-qPCR kit reverse-transcribes RNA_{total} to cDNA during RT-qPCR whilst the WT-Ovation™ Pico RNA Amplification System reverse transcribes RNA_{total} to cDNA prior to RT-qPCR. The iScript one-step RT-qPCR kit requires 10ng of RNA_{total} per reaction whilst the WT-Ovation™ Pico RNA Amplification System requires 10ng of cDNA per RT-qPCR reaction. The WT-Ovation™ Pico RNA Amplification System proved to maximise the experimental potential of a single human dental pulp by 161 – times (in technical duplicate).

Using an input of 10 ng cDNA per RT-qPCR reaction, it was shown that 398 RT-qPCR reactions could be performed, in technical duplicate, from a single reverse-transcription-amplification reaction. We further calculated that a total of 16 reverse-transcription-amplification reactions could be performed from the above mean (± SEM) yield (ng) of

extracted pulp RNA_{total}; expanding the experimental potential of a single pulp tissue from 40 RT-qPCR reactions using the iScript one-step RT-qPCR kit to a possible 6,441 RT-qPCR reactions, when carried out in technical duplicate with the WT-Ovation™ Pico RNA Amplification System.

The WT-Ovation™ Pico RNA Amplification System was discovered only after all RT-qPCR had been performed for the reference gene stability analysis and the mRNA expression analysis of angiogenic growth factors in this experimental chapter. It was found to be the most appropriate method for reverse-transcribing human dental pulp RNA_{total} to cDNA for the purpose of RT-qPCR analysis and the remaining volumes of RNA_{total} isolated from the pulps treated with an orthodontic force for 2 weeks prior to tooth extraction were reverse-transcribed to cDNA in order to expand the study in **Chapter 4**.

3.4.4 Normalising to total RNA and Distribution of Reference Gene Expression

In order to examine the range of expression of the reference genes in our control and treated samples, RT-qPCR was performed in technical duplicate for each gene with the iScript one-step RT-qPCR kit, inputting an equal mass of RNA_{total} (10 ng) per reaction; C_q values were compared directly.

A paired Student's t-Test was performed on C_q values to determine the stability of expression of each reference gene in the treated samples (n = 10) relative to the contralateral controls (n = 10); no statistically significant difference (p > 0.05) was identified (**Table 22**).

Gene	P-value
PPIA	0.52
GAPDH	0.44
ACTB	0.20
HPRT1	0.08
RPL13A	0.08
UBC	0.08
YWHAZ	0.08

Table 22 Normalisation of candidate reference gene expression against total RNA

To identify differences in candidate gene expression between the control (n = 10) and treated (n = 10) subgroups, a paired Student's t-Test was implemented. There was no significant difference ($p > 0.05$) in expression of any of the candidate genes between the control and treated subgroups.

C_q values were plotted on box-whisker plots to show the distribution of gene expression across the sample set (**Figure 7**). C_q values for HPRT1 and PPIA were higher than those for the remainder of the panel, indicating that there were lower amounts of template present for these genes in the pulp samples; as C_q values are inversely correlated to the amount of template present for the gene of interest (GOI) in the original sample (Heid *et al.* 1996).

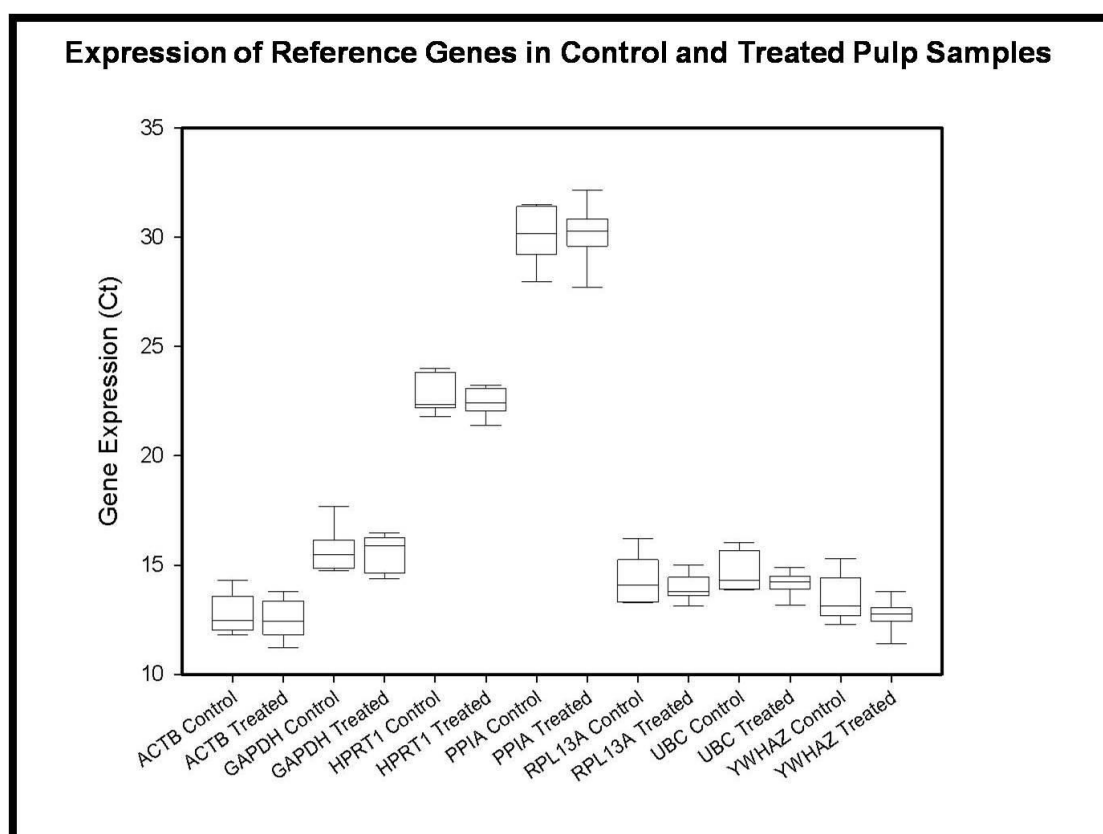


Figure 7 Distribution of candidate reference gene expression in control and treated samples

Box-Whisker plots showing distribution of gene expression according to C_q values for control ($n = 10$) and treated ($n = 10$) subgroups. Whiskers correspond to data range while boxes illustrate the 25th percentile, median value and 75th percentiles. A paired Student's t -Test showed no significant difference ($p > 0.05$) in mRNA expression of candidate genes between control and treated subgroups.

3.4.5 Analysis of Reference Gene Expression Stability – *BestKeeper*©

C_q values ($n = 20$) were inserted into the *BestKeeper*© Microsoft Excel based tool and the geometric mean, arithmetic mean, minimal value, maximal value, standard deviation (SD) and coefficient of variation (CV) were calculated for each reference gene (**Table 23**). CV values for HPRT1 and PPIA were lower than those of the other reference genes, indicating that these genes are not directly comparable with the others (Pfaffl *et al.* 2004). HPRT1 and PPIA were excluded from further stability analyses. The remaining genes were ranked according to stability, indicated by SD values, from

most to least stable, with the most stable gene having the lowest variability: (1) UBC, (2) RPL13A, (3) GAPDH, (4) ACTB and YWHAZ.

	ACTB	GAPDH	HPRT1	PPIA	RPL13A	UBC	YWHAZ
CV	5.66	4.17	2.50	2.98	4.45	3.67	5.46
SD	0.71	0.65	0.56	0.90	0.64	0.53	0.71
Gene ranking according to stability: (1) UBC; (2) RPL13A; (3) GAPDH; (4) ACTB and YWHAZ							

Table 23 Analysis of variance and stability of each candidate reference gene using the *BestKeeper*® tool

Coefficient of variation (CV) and standard deviations (SD) as calculated by the *BestKeeper*® tool. HPRT1 and PPIA were shown to have a different variance to the remaining candidate genes. Whilst a low variance is considered to be optimal, C_q values for these two genes were greater ($C_q = 39 - < 45$, **Chapter 8, section 8.1.8.1 Table 57 – 58**) than the remaining candidate genes and the genes of interest, indicating that these genes are expressed in low abundance within human dental pulps. Therefore these two genes were excluded from further stability analysis. SD values were used to rank genes according to stability where the lowest variation corresponds with the most stable gene across the sample set ($n = 20$).

By performing numerous pair-wise correlation analyses and combining highly correlated genes into an index against which all remaining genes were compared by regression analysis, *BestKeeper*® identified RPL13A, UBC and YWHAZ to be the most tightly correlated genes in the panel (**Table 24**).

Repeated pair-wise correlation analysis (n = 20)					
	ACTB	GAPDH	RPL13A	UBC	YWHAZ
vs.	Reference	Reference	Reference	Reference	Reference
	Gene 1	Gene 2	Gene 3	Gene4	Gene 5
Reference Gene 2	0.247	-	-	-	-
<i>p</i> - Value	0.294	-	-	-	-
Reference Gene 3	0.589	0.640	-	-	-
<i>p</i> - Value	0.006	0.002	-	-	-
Reference Gene 4	0.541	0.570	0.935	-	-
<i>p</i> - Value	0.014	0.009	0.001	-	-
Reference Gene 5	0.627	0.595	0.865	0.913	-
<i>p</i> - Value	0.003	0.006	0.001	0.001	-
BestKeeper® vs.	ACTB	GAPDH	RPL13A	UBC	YWHAZ
Coeff. of corr. [r]	0.728	0.699	0.944	0.928	0.947
<i>p</i> - Value	0.001	0.001	0.001	0.001	0.001
Repeated pair-wise correlation analysis (n = 20)					
Reference Gene vs. <i>BestKeeper</i> ® index out of 5					
Reference Gene	Reference	Reference	Reference	Reference	Reference
	Gene 1	Gene 2	Gene 3	Gene 4	Gene 5
	ACTB	GAPDH	RPL13A	UBC	YWHAZ
Coeff. of corr. [r]	0.728	0.699	0.944	0.928	0.947
Coeff. of det. [r ²]	0.530	0.489	0.891	0.861	0.897
<i>p</i> - Value	0.001	0.001	0.001	0.001	0.001

Table 24 Analysis of inter-reference gene relations using pair-wise correlation and regression analysis in the *BestKeeper* © Excel-based tool

Repeated pair-wise correlation analysis as calculated by the *BestKeeper*® tool. Candidate genes were initially correlated with one another and then correlated against the *BestKeeper Index*. Coefficient of correlation (*r*), coefficient of determination (*r*²) and *p*-values were calculated. Significance was given at (*p* < 0.05).

3.4.6 Analysis of Reference Gene Expression Stability – geNorm and NormFinder

Gene expression stability was assessed by the geNorm and NormFinder algorithms by inputting C_q values into GenEx software version 4.3.5. geNorm calculated average pair-wise variations and used a step-wise exclusion process to identify a pair of constitutively expressed genes; genes were ranked by an assigned gene expression stability value (M) (**Figure 8**). ACTB was shown to be the least stable candidate while RPL13A and UBC were identified as the most stably expressed pair of genes under our experimental conditions.

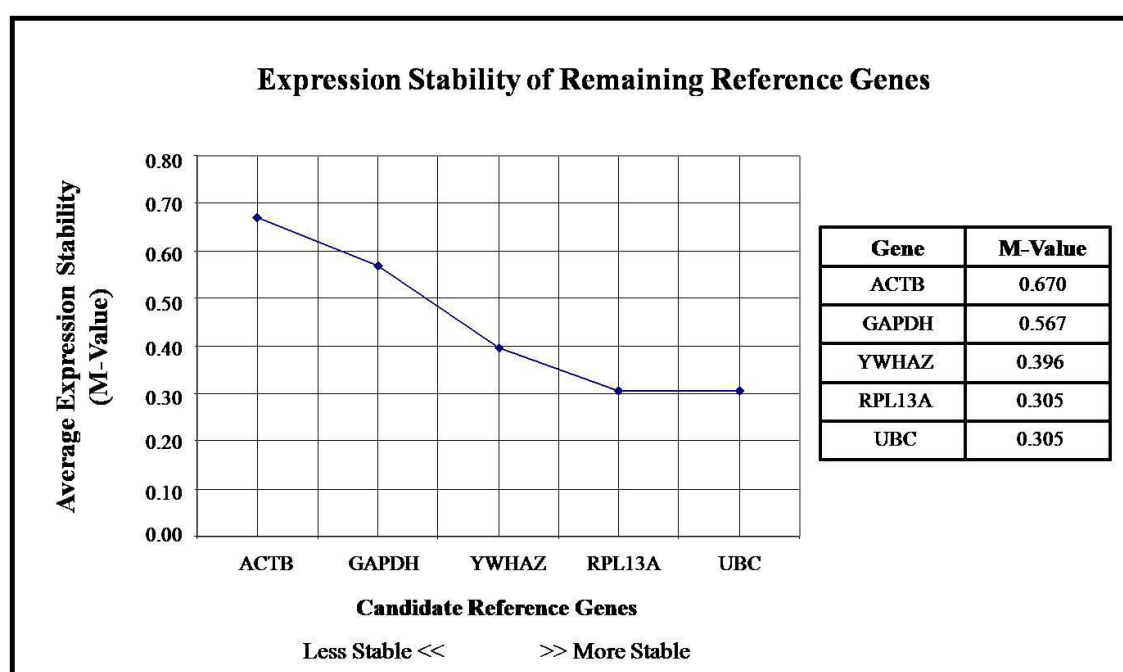


Figure 8 Analysis of expression stability of candidate reference genes using the geNorm algorithm

Plot of gene expression stability (M) values, average pair-wise variation in mRNA expression of a single candidate gene relative to variation in mRNA expression of the remaining candidate genes, illustrates ACTB to be the least stable candidate gene and RPL13A and UBC to be the most stable pair of candidate genes across whole sample set (n = 20).

The NormFinder algorithm determined the stability of individual candidate genes in inter- and intra-group analyses; and genes were ranked according to decreasing variability. Inter-group analyses (n = 20) identified ACTB to be the least stable and UBC to be the most stable reference gene under this experimental system (**Table 25**).

Gene	Variability
ACTB	0.732
GAPDH	0.712
YWHAZ	0.365
RPL13A	0.229
UBC	0.204
Best Gene UBC	Variability 0.204

Table 25 Analysis of expression stability of candidate reference genes using the NormFinder algorithm – without groups

Reference genes ranked according to NormFinder analysis with GenEx software version 4.3.5 when considering control and treated samples as one group (n = 20). UBC identified as the most stable gene among samples with the lowest variability.

While inter- and intra-group analyses of control (n = 10) and treated (n = 10) subgroups indicated GAPDH to be the least stable and UBC to most stable gene from the panel (**Figure 9**). NormFinder further identified that the geometric mean of UBC and RPL13A demonstrated the lowest amount of variability overall and should be used as a normalisation factor in place of a single gene. Both analyses of the full data set (n = 20) and the subgroups (n = 10) identified UBC as the single most stable reference gene under our experimental system.

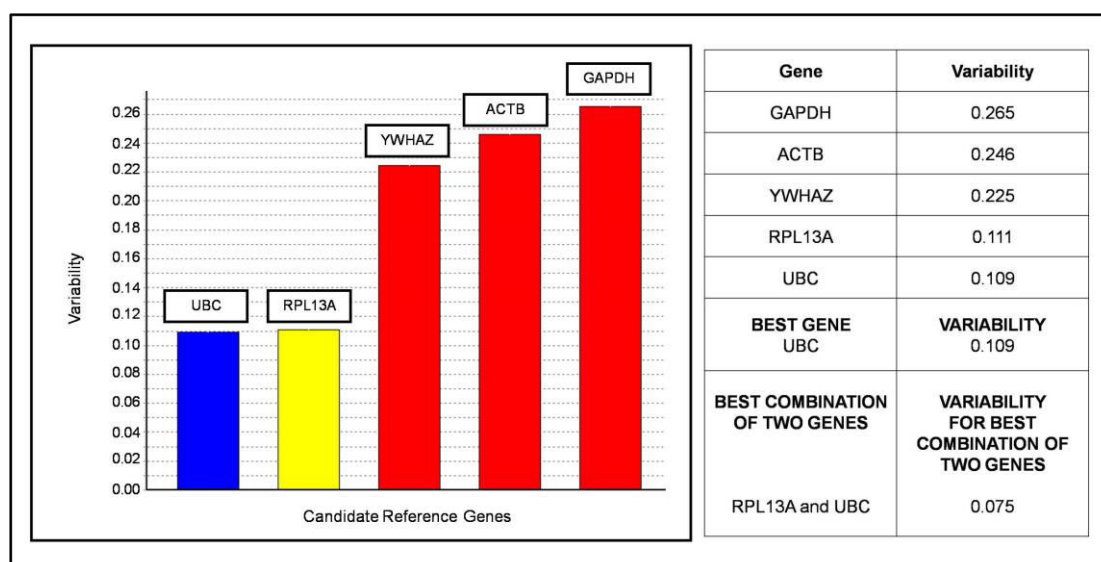


Figure 9 Analysis of expression stability of candidate reference genes using the NormFinder algorithm: Intra-group Variation

Plot of candidate reference genes ranked according to stability when inter- and intra-group variation is considered by NormFinder in GenEx software version 4.3.5. UBC was found to be the most stable among the candidate genes, with the lowest variability in expression among control (n =10) and treated (n = 10) subgroups. When used in combination, the mean expression of UBC and RPL13A was shown to have the lowest overall variability.

3.4.7 Expression of Angiogenic Growth Factors in Human Dental Pulps

3.4.7.1 Confirming the Expression of Angiogenic Growth Factor mRNA in Human Dental Pulps and MG-63 Osteosarcoma Cells

RT-PCR was performed using primers for the angiogenesis-specific growth factors FGF2, PDGFA, TGFB1 and VEGFA (**Table 3 – Table 6**), RNA from MG-63 osteosarcoma cells and RNA previously isolated from a control pulp sample (n = 1, section **3.4.2.1**). Gel electrophoresis was used to separate RT-PCR products (**Figure 10**). MG-63 osteosarcoma cells were found to express similar levels of angiogenic growth factor mRNA as the human dental pulp, relative to GAPDH. As qualitative analysis showed little difference between mRNA expression levels in an

aggressive cancer cell line and the human dental pulp, a quantitative method (RT-qPCR) was deemed more appropriate for the study.

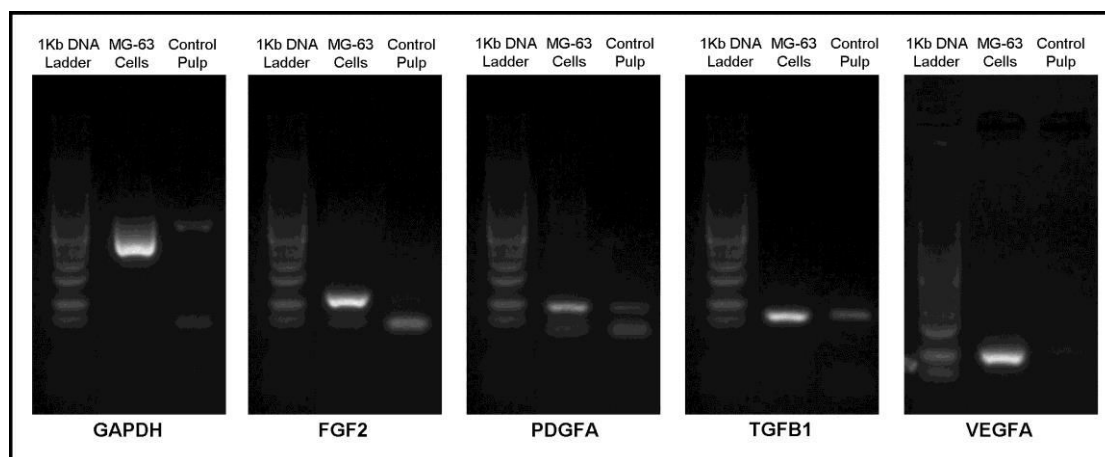


Figure 10 Gel electrophoretograms of RT-PCR products in the MG-63 osteosarcoma cell line and an untreated human dental pulp

3.4.7.2 Determining the Relative Expression of Angiogenic Growth Factor mRNA in Human Dental Pulps

RT-qPCR was performed in technical duplicate, using primers for the angiogenic growth factors FGF2, PDGFA, TGFB1 and VEGFA (**Table 3 – Table 8**) on pulp samples isolated from premolar teeth that had been treated with an orthodontic force for two weeks prior to extraction (n = 10) and on untreated contralateral ‘controls’ (n = 10).

3.4.7.2.a Comparing mRNA Expression using the Livak Comparative Ct Method

C_q values were input into Microsoft Excel® spreadsheet software in order to perform Delta C_t and Delta-Delta C_t analysis as outlined by Livak and Schmittgen *et al.* (2001) and clarified in 2008 (Schmittgen and Livak 2008).

Data was normalised against: (1) UBC (**Table 26 – Table 29**) or (2) the geometric mean of UBC and RPL13A (**Table 30 – Table 33**).

Delta Ct Method			
Log Transformed Delta Ct (2 ^{-ΔCt}):			
Control 1	1.930E-02	Treated 1	2.225E-02
Control 2	4.025E-02	Treated 2	3.955E-02
Control 3	2.768E-02	Treated 3	5.852E-02
Control 4	2.836E-02	Treated 4	2.610E-02
Control 5	4.544E-02	Treated 5	4.109E-02
Control 6	2.956E-02	Treated 6	4.419E-02
Control 7	3.492E-02	Treated 7	5.024E-02
Control 8	3.202E-02	Treated 8	3.224E-02
Control 9	2.047E-02	Treated 9	4.328E-02
Control 10	5.165E-02	Treated 10	3.901E-02
Mean ± SD	3.297E-02 ± 1.036E-02	Mean ± SD	3.965E-02 ± 1.078E-02
CV (%)	3.143E+01	CV (%)	2.719E+01
Fold Change Between Control and Treated:		1.203E+00	
Fold Change Due to Treatment:		-0.831	

Delta Delta Ct Method			
FGF2			
Control		Treated	
Mean ± SD	1.960E+01 ± 9.541E-01	Mean ± SD	1.887E+01 ± 4.614E-01
CV (%)	4.869E+00	CV (%)	2.446E+00
UCB			
Control		Treated	
Mean ± SD	1.461E+01 ± 8.788E-01	Mean ± SD	1.416E+01 ± 5.190E-01
CV (%)	6.015E+00	CV (%)	3.665E+00
Delta Ct (ΔC _t) - (Target Gene - Reference Gene):			
Control	4.988E+00	Treated	4.709E+00
Delta Delta Ct (ΔΔC _t) - (Treated - Control):		-2.785E-01	
Log Transformed Delta Delta Ct (2 ^{-ΔΔCt}):		1.213E+00	
Fold Change Due to Treatment:		-0.824	

Table 26 Delta C_t and Delta-Delta C_t analysis of angiogenic growth factor FGF2 normalised against the reference gene UBC

Delta Ct Method			
Log Transformed Delta Ct (2 ^{-ΔCt}):			
Control 1	4.889E-05	Treated 1	4.756E-05
Control 2	3.159E-05	Treated 2	2.150E-05
Control 3	2.479E-05	Treated 3	4.546E-05
Control 4	6.297E-05	Treated 4	6.939E-05
Control 5	6.496E-05	Treated 5	3.010E-05
Control 6	2.917E-05	Treated 6	4.940E-05
Control 7	4.468E-05	Treated 7	7.809E-05
Control 8	2.629E-05	Treated 8	2.602E-05
Control 9	5.132E-05	Treated 9	6.843E-05
Control 10	4.872E-05	Treated 10	4.723E-05
Mean ± SD	4.334E-05 ± 1.473E-05	Mean ± SD	4.832E-05 ± 1.913E-05
CV (%)	3.398E+01	CV (%)	3.959E+01
Fold Change Between Control and Treated:		1.115E+00	
Fold Change Due to Treatment:		-0.897	

Delta Delta Ct Method			
PDGFA			
Control		Treated	
Mean ± SD	2.918E+01 ± 9.484E-01	Mean ± SD	2.861E+01 ± 8.091E-01
CV (%)	3.250E+00	CV (%)	2.828E+00
UBC			
Control		Treated	
Mean ± SD	1.461E+01 ± 8.786E-01	Mean ± SD	1.416E+01 ± 5.190E-01
CV (%)	6.015E+00	CV (%)	3.665E+00
Delta Ct (ΔC _t) - (Target Gene - Reference Gene):			
Control	1.457E+01	Treated	1.445E+01
Delta Delta Ct (ΔΔC _t) - (Treated - Control):		-1.230E-01	
Log Transformed Delta Delta Ct (2 ^{-ΔΔC_t}):		1.089E+00	
Fold Change Due to Treatment:		-0.918	

Table 27 Delta C_t and Delta-Delta C_t analysis of angiogenic growth factor PDGFA normalised against the reference gene UBC

Delta Ct Method				Delta Delta Ct Method			
Log Transformed Delta Ct (2 ^{-ΔCt}):				TGFB1			
Control 1	4.359E-02	Treated 1	3.315E-02	Control		Treated	
Control 2	3.955E-02	Treated 2	2.956E-02	Mean ± SD	1.895E+01 ± 8.823E-01	Mean ± SD	1.868E+01 ± 8.471E-01
Control 3	5.256E-02	Treated 3	6.272E-02	CV (%)	4.657E+00	CV (%)	4.536E+00
Control 4	7.829E-02	Treated 4	8.717E-02	UBC			
Control 5	6.745E-02	Treated 5	3.678E-02	Control		Treated	
Control 6	4.167E-02	Treated 6	4.528E-02	Mean ± SD	1.461E+01 ± 8.788E-01	Mean ± SD	1.416E+01 ± 5.190E-01
Control 7	2.435E-02	Treated 7	3.729E-02	CV (%)	6.015E+00	CV (%)	3.665E+00
Control 8	2.547E-02	Treated 8	3.408E-02	Delta Ct (ΔC _t) - (Target Gene - Reference Gene):			
Control 9	7.032E-02	Treated 9	4.671E-02	Control	4.338E+00	Treated	4.518E+00
Control 10	1.005E-01	Treated 10	4.753E-02	Delta Delta Ct (ΔΔC _t) - (Treated - Control):			
Mean ± SD	5.437E-02 ± 2.441E-02	Mean ± SD	4.603E-02 ± 1.737E-02	Log Transformed Delta Delta Ct (2 ^{-ΔΔCt}):			
CV (%)	4.489E+01	CV (%)	3.774E+01	Fold Change Due to Treatment:			
Fold Change Between Control and Treated:		8.465E-01		-1.132			
Fold Change Due to Treatment:		-1.181					

Table 28 Delta C_t and Delta-Delta C_t analysis of angiogenic growth factor TGFB1 normalised against the reference gene UBC

Delta Ct Method				Delta Delta Ct Method			
Log Transformed Delta Ct (2 ^{-ΔCt}):				VEGFA			
Control 1	1.960E-03	Treated 1	2.339E-03	Control		Treated	
Control 2	1.480E-03	Treated 2	1.480E-03	Mean ± SD	2.367E+01 ± 9.793E-01	Mean ± SD	2.303E+01 ± 4.994E-01
Control 3	9.971E-04	Treated 3	1.791E-03	CV (%)	4.138E+00	CV (%)	2.168E+00
Control 4	2.371E-03	Treated 4	1.887E-03	UBC			
Control 5	3.448E-03	Treated 5	2.072E-03	Control		Treated	
Control 6	1.559E-03	Treated 6	2.613E-03	Mean ± SD	1.461E+01 ± 8.788E-01	Mean ± SD	1.416E+01 ± 5.190E-01
Control 7	1.893E-03	Treated 7	3.023E-03	CV (%)	6.015E+00	CV (%)	3.665E+00
Control 8	1.586E-03	Treated 8	1.592E-03	Delta Ct (ΔC _t) - (Target Gene - Reference Gene):			
Control 9	1.880E-03	Treated 9	2.396E-03	Control	9.058E+00	Treated	8.877E+00
Control 10	2.595E-03	Treated 10	2.604E-03	Delta Delta Ct (ΔΔC _t) - (Treated - Control):			
Mean ± SD	1.977E-03 ± 6.874E-04	Mean ± SD	2.180E-03 ± 4.982E-04	Log Transformed Delta Delta Ct (2 ^{-ΔΔCt}):			
CV (%)	3.477E+01	CV (%)	2.286E+01	Fold Change Due to Treatment:			
Fold Change Between Control and Treated:		1.102E+00		-0.882			
Fold Change Due to Treatment:		-0.907					

Table 29 Delta C_t and Delta-Delta C_t analysis of angiogenic growth factor VEGFA normalised against the reference gene UBC

Delta Ct Method				Delta Delta Ct Method			
Log Transformed Delta Ct (2 ^{-ΔCt}):				FGF2			
Control 1	2.002E-02	Treated 1	1.985E-02	Control		Treated	
Control 2	3.684E-02	Treated 2	3.801E-02	Mean ± SD	1.960E+01 ± 9.541E-01	Mean ± SD	1.887E+01 ± 4.614E-01
Control 3	3.286E-02	Treated 3	5.375E-02	CV (%)	4.869E+00	CV (%)	2.446E+00
Control 4	2.300E-02	Treated 4	2.578E-02	GEOMEAN UBC & RPL13A			
Control 5	4.060E-02	Treated 5	3.814E-02	Control		Treated	
Control 6	2.431E-02	Treated 6	4.004E-02	Mean ± SD	1.445E+01 ± 9.576E-01	Mean ± SD	1.407E+01 ± 5.359E-01
Control 7	2.881E-02	Treated 7	3.935E-02	CV (%)	6.625E+00	CV (%)	3.810E+00
Control 8	2.619E-02	Treated 8	3.103E-02	Delta Ct (ΔC _t) - (Target Gene - Reference Gene):			
Control 9	1.680E-02	Treated 9	4.520E-02	Control	5.141E+00	Treated	4.801E+00
Control 10	4.786E-02	Treated 10	4.011E-02	Delta Delta Ct (ΔΔC _t) - (Treated - Control):			
Mean ± SD	2.973E-02 ± 9.757E-03	Mean ± SD	3.713E-02 ± 9.604E-03	Log Transformed Delta Delta Ct (2 ^{-ΔΔCt}):			
CV (%)	3.282E+01	CV (%)	2.587E+01	Fold Change Due to Treatment:			
Fold Change Between Control and Treated:		1.249E+00		Log Transformed Delta Delta Ct (2 ^{-ΔΔCt}):		1.268E+00	
Fold Change Due to Treatment:		-0.801		Fold Change Due to Treatment:		-0.790	

Table 30 Delta C_t and Delta-Delta C_t analysis of angiogenic growth factor FGF2 normalised against the geometric mean expression of the reference genes RPL13A and UBC

Delta Ct Method				Delta Delta Ct Method			
Log Transformed Delta Ct (2 ^{-ΔCt}):				PDGFA			
Control 1	5.071E-05	Treated 1	4.242E-05	Control		Treated	
Control 2	2.892E-05	Treated 2	2.066E-05	Mean ± SD	2.918E+01 ± 9.484E-01	Mean ± SD	2.861E+01 ± 8.091E-01
Control 3	2.943E-05	Treated 3	4.176E-05	CV (%)	3.250E+00	CV (%)	2.828E+00
Control 4	5.108E-05	Treated 4	6.855E-05	GEOMEAN UBC & RPL13A			
Control 5	5.804E-05	Treated 5	2.794E-05	Control		Treated	
Control 6	2.399E-05	Treated 6	4.476E-05	Mean ± SD	1.445E+01 ± 9.576E-01	Mean ± SD	1.407E+01 ± 5.359E-01
Control 7	3.686E-05	Treated 7	6.114E-05	CV (%)	6.625E+00	CV (%)	3.810E+00
Control 8	2.150E-05	Treated 8	2.505E-05	Delta Ct (ΔC _t) - (Target Gene - Reference Gene):			
Control 9	4.212E-05	Treated 9	7.146E-05	Control	1.473E+01	Treated	1.454E+01
Control 10	4.515E-05	Treated 10	4.856E-05	Delta Delta Ct (ΔΔC _t) - (Treated - Control):			
Mean ± SD	3.878E-05 ± 1.257E-05	Mean ± SD	4.523E-05 ± 1.772E-05	Log Transformed Delta Delta Ct (2 ^{-ΔΔCt}):			
CV (%)	3.241E+01	CV (%)	3.918E+01	Fold Change Due to Treatment:			
Fold Change Between Control and Treated:		1.166E+00		Log Transformed Delta Delta Ct (2 ^{-ΔΔCt}):		1.137E+00	
Fold Change Due to Treatment:		-0.857		Fold Change Due to Treatment:		-0.880	

Table 31 Delta C_t and Delta-Delta C_t analysis of angiogenic growth factor PDGFA normalised against the geometric mean expression of the reference genes RPL13A and UBC

Delta Ct Method				Delta Delta Ct Method			
Log Transformed Delta Ct ($2^{-\Delta C_t}$):				TGFB1			
Control 1	4.520E-02	Treated 1	2.958E-02	Control		Treated	
Control 2	3.621E-02	Treated 2	2.841E-02	Mean \pm SD	1.895E+01 \pm 8.823E-01	Mean \pm SD	1.868E+01 \pm 8.471E-01
Control 3	6.239E-02	Treated 3	5.761E-02	CV (%)	4.657E+00	CV (%)	4.538E+00
Control 4	6.348E-02	Treated 4	8.612E-02	GEOMEAN UBC & RPL13A			
Control 5	6.027E-02	Treated 5	3.414E-02	Control		Treated	
Control 6	3.426E-02	Treated 6	4.102E-02	Mean \pm SD	1.445E+01 \pm 9.578E-01	Mean \pm SD	1.407E+01 \pm 5.359E-01
Control 7	2.009E-02	Treated 7	2.921E-02	CV (%)	6.625E+00	CV (%)	3.810E+00
Control 8	2.083E-02	Treated 8	3.280E-02	Delta Ct (ΔC_t) - (Target Gene - Reference Gene):			
Control 9	5.771E-02	Treated 9	4.878E-02	Control	4.492E+00	Treated	4.608E+00
Control 10	9.310E-02	Treated 10	4.887E-02	Delta Delta Ct ($\Delta\Delta C_t$) - (Treated - Control):			
Mean \pm SD	4.935E-02 \pm 2.251E-02	Mean \pm SD	4.635E-02 \pm 1.797E-02	Log Transformed Delta Delta Ct ($2^{-\Delta\Delta C_t}$):			
CV (%)	4.561E+01	CV (%)	4.117E+01	Fold Change Due to Treatment:			
Fold Change Between Control and Treated:		8.845E-01		Fold Change Due to Treatment:			
Fold Change Due to Treatment:		-1.131		Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			
				Fold Change Due to Treatment:			

shown to be no significant difference in analysing samples as individuals by the Delta C_t method when compared to the Delta-Delta C_t method which analyses samples in their respective contralateral pairs ($p = 0.83$).

3.4.7.2.b **Comparing mRNA Expression using Pfaffl's REST Method**

C_q values from the pulp samples and reaction efficiencies, determined from the MG-63 osteosarcoma RNA_{total} standard curves, were entered into the REST-2009© software and expression ratios were calculated normalising against: (1) UBC (**Figure 11**) or (2) the geometric mean of RPL13A and UBC (**Figure 12**).

It was shown that there was no significant up- or down-regulation of mRNA expression of the growth factors FGF2, PDGFA, TGFB1 and VEGFA in human dental pulps treated with an orthodontic force for a period of two weeks when data was normalised against UBC only (**Figure 11**). When data was normalised against the geometric mean of RPL13A and UBC, FGF2 was found to be significantly up-regulated by a mean factor of 1.383 (**Figure 12**).

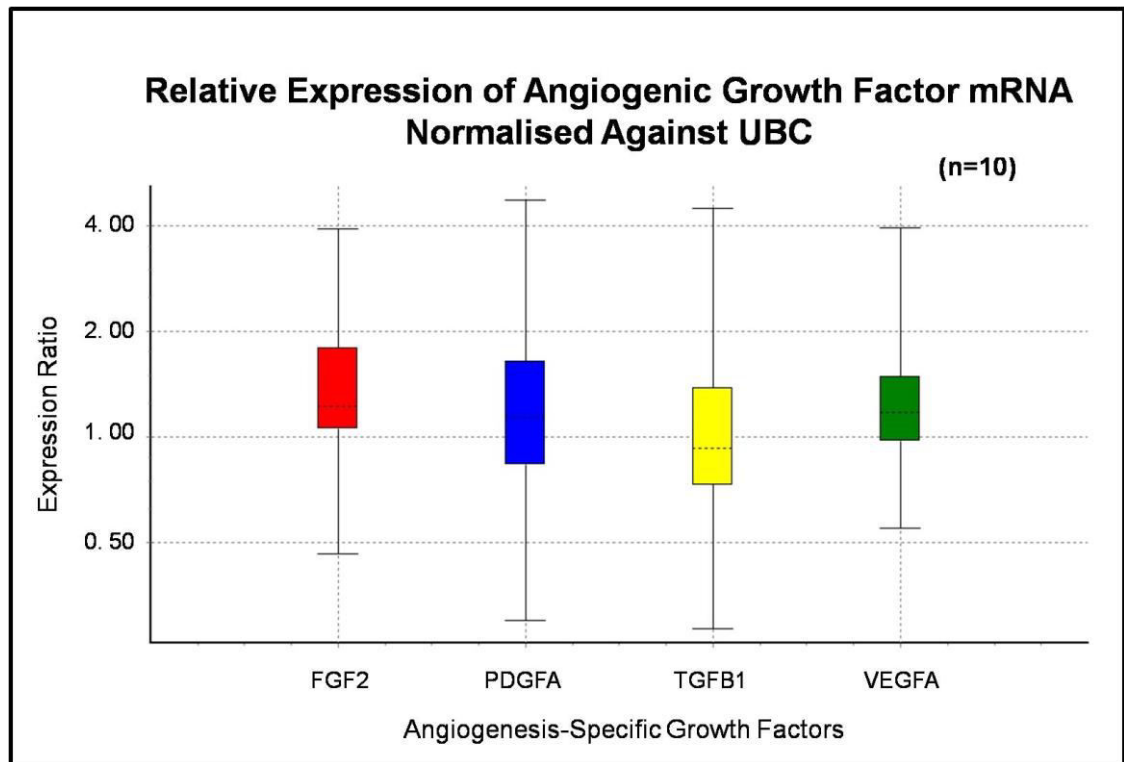


Figure 11 Differential expression analysis of angiogenic growth factor mRNA in two week treated samples relative to contralateral controls. Normalising against UBC.

Plot shows expression ratios calculated by REST-2009© software. Box-Whisker plots illustrate the range of observations (whiskers), 25th and 75th percentiles (boxes) and sample medians (dashed line). Data was normalised against UBC, which has been identified as the most appropriate individual reference gene under our experimental conditions. There was no significant difference ($p > 0.05$) in mRNA expression of any of the growth factors in the two week treated samples ($n = 10$) relative to the contralateral controls ($n = 10$).

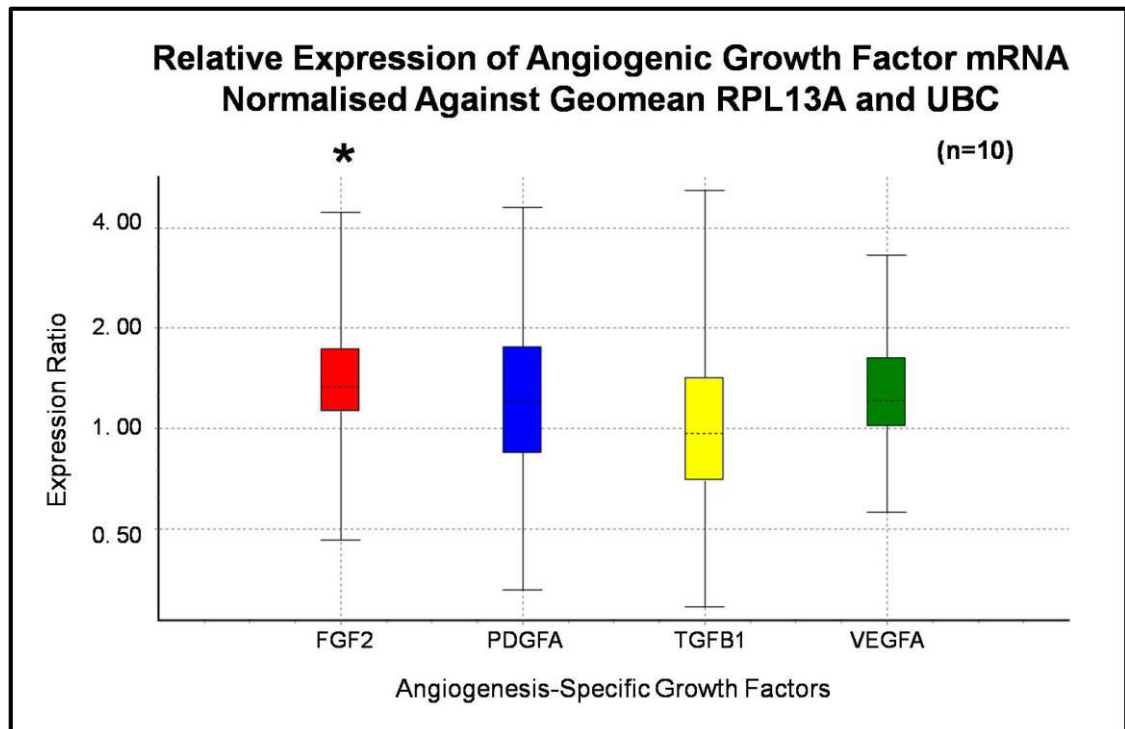


Figure 12 Differential expression analysis of angiogenic growth factor mRNA in two week treated samples relative to contralateral controls. Normalising against the geometric mean of RPL13A and UBC.

Plot shows expression ratios calculated by REST-2009© software. Box-Whisker plots illustrate the range of observations (whiskers), 25th and 75th percentiles (boxes) and sample medians (dashed line). Data was normalised against the geometric mean of RPL13A and UBC, which has been identified as a suitable normalisation factor under our experimental conditions. There was a significant up-regulation of FGF2 mRNA expression in the two week treated samples (n = 10) relative to the contralateral controls (n = 10)

* indicates statistical significance (p < 0.05).

Expression ratios were subsequently calculated by normalising against the full panel of candidate reference genes and it was identified that by normalising against generic reference genes, a number of erroneous interpretations could be made (**Figure 13**).

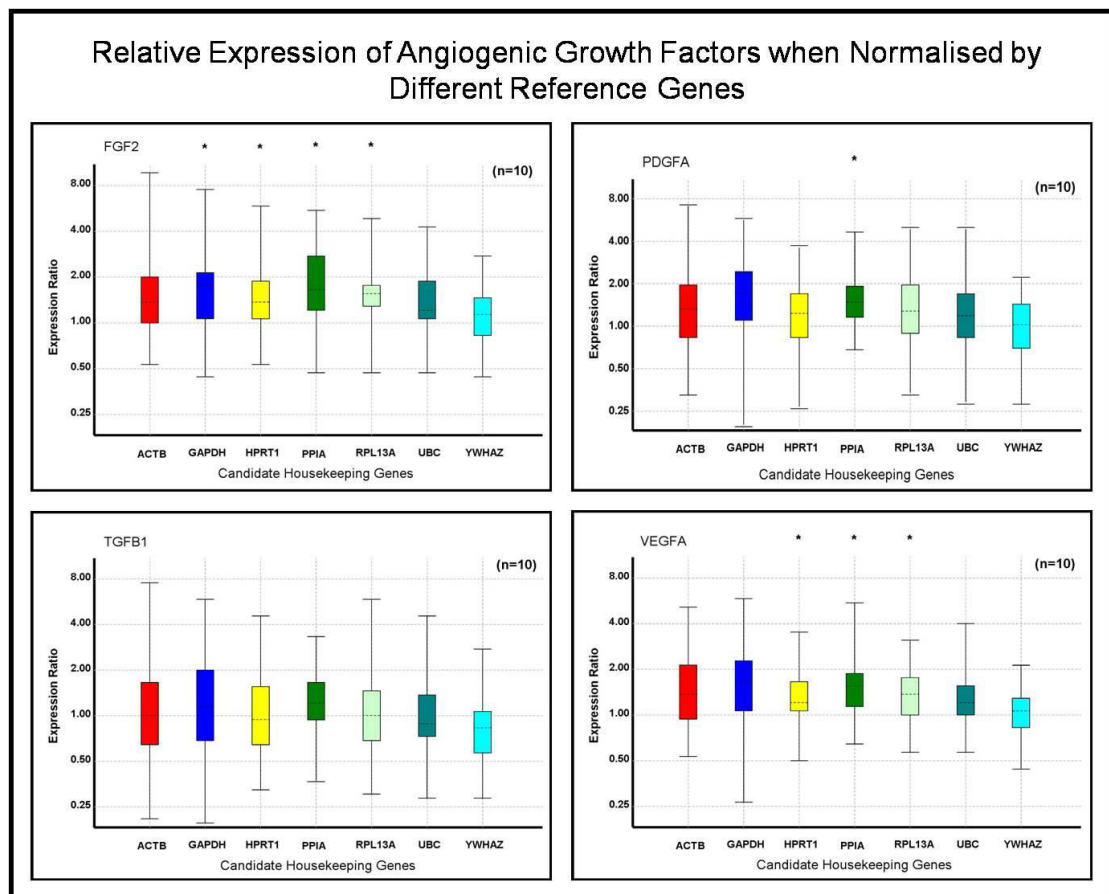


Figure 13 Normalisation of differential expression analysis data with multiple reference genes

Box-Whisker plots illustrate the range of observations (whiskers), 25th and 75th percentiles (boxes) and sample medians (dashed line). FGF2, PDGFA and VEGFA mRNA expression was significantly up-regulated in treated samples (n = 10) relative to contralateral controls (n = 10) when normalised against individual reference genes that had been identified as being unstable, demonstrating that identification of a stable reference gene is fundamental. * indicates statistical significance (p < 0.05).

FGF2 was shown to be significantly up-regulated in treated samples relative to contralateral controls when data was normalised with GAPDH, HPRT1, PPIA or RPL13A (p < 0.05). PDGFA was significantly up-regulated when data was normalised with PPIA (p < 0.05). TGFβ1 showed no significant difference between expression of mRNA in control and treated samples (p > 0.05). VEGFA was significantly up-regulated when sample data was normalised against HPRT1, PPIA or RPL13A (p < 0.05).

3.5 Discussion

Previous studies on pulp-derived stem cells and pulp tissues, using RT-qPCR to analyse differential gene expression, have been carried out using generic reference genes for normalising data (Dissanayaka *et al.* 2015, Dong *et al.* 2013, Gronthos *et al.* 2002, Liu *et al.* 2007, McLachlan *et al.* 2005). As it is known that gene expression undergoes modification under changing experimental conditions and while reference genes are expressed constitutively among cell types, modification of their regulation cannot be ignored.

Orthodontic treatment is known to be associated with a number of reversible and irreversible alterations to the tooth and dental pulp (Bauss *et al.* 2009, Hamersky *et al.* 1980, Ramazanzadeh *et al.* 2009, Sano *et al.* 2002, Santamaria *et al.* 2006, Stenvik and Mjor 1970). As there is possible risk of damage to the pulp during routine patient treatment, characterisation of the pulp's responses to these heavy forces is important; as this may provide an insight into the mechanisms involved and could lead to clinical interventions.

In order to ensure that any changes in gene expression, as a result of experimental manipulation or physiological alteration, are measured accurately; it is essential that a stable reference gene is identified for unique experimental systems. This experimental chapter has focussed on the standardisation of experimental protocols from sample collection and RNA_{total} quantification through to validation of reference gene stability and quantification of differential mRNA expression.

3.5.1 Sample Storage and RNA Preservation

While the use of snap- or flash-freezing tissue samples in liquid nitrogen is a recognised approach for ensuring sample integrity and RNA quality, safety hazards associated with

the technique make its use impractical in clinical environments and in transporting samples (<http://stores.biochem.uiowa.edu/Pages/ln2msds.htm>). Past work on human tumour cells lines, ocular tissues, urine cell pellets and other tissues used in clinical diagnostics have identified that samples stored in an RNA preservative such as RNALater™ will yield similar amounts (ng) of RNA_{total} as samples of a similar weight that have been snap-frozen (Florell *et al.* 2001, Grotzer *et al.* 2000, Medeiros *et al.* 2003, Wang *et al.* 2001). We have shown that there is no statistically significant difference in the amount (ng) of RNA attainable from human dental pulps stored in RNALater™, when compared with snap-frozen samples ($p > 0.05$). Our findings substantiate those of past studies (Florell *et al.* 2001, Grotzer *et al.* 2000, Medeiros *et al.* 2003, Wang *et al.* 2001) in that RNA preservation solutions ensure minimal RNA degradation, are safe for use in clinical environments and make transportation of samples less onerous.

3.5.2 RNA Isolation, Yield and Sample Purity

3.5.2.1 RNA Isolation Protocols

Considered selection of the tissue/cell homogenisation and RNA isolation protocols used in any experiment is critical as these two frequently overlooked stages determine the quality and quantity of RNA_{total} isolated from a sample. In clinical studies where valuable samples are under investigation, any loss or degradation of RNA_{total} could prevent accurate assessment of the state of the tissue and could lead to erroneous conclusions being made (Bartlett 2002). It was noted that homogenisation of the tissue by a mortar and pestle in liquid nitrogen resulted in a loss of sample. Fractions of the sample were projected out of the mortar as the liquid nitrogen reacted with the ambient temperature; or the homogenised tissue, now in powder form, stuck to the pestle and could not easily be transferred into an RNA stabilisation solution prior to defrosting

completely, thus compromising sample integrity. Although the amount of sample that was lost could not be quantified, it is fundamental that when working with valuable samples of small mass (± 0.05 g ($n = 5$)), restrictions are put in place to prevent sample loss and protect sample integrity. We therefore suggest that this method of homogenisation is discounted as an appropriate technique for use with the human dental pulp. Sample loss was also noted when tissue was passed through a needle in an RNA stabilisation solution. Initially the sample got stuck inside the needle's shaft as it was too large to pass through unhindered; large amounts of pressure were then used to force the sample back out into the collection vessel. Due to the build up in pressure in the needle shaft, the sample was rapidly ejected, resulting in spillage. As the sample broke down and was able to pass through the needle with ease, collagenous fibres built up and obstructed the needle shaft, preventing complete homogenisation of the tissue. As samples cannot be homogenised completely with this technique, we propose that this is an unsuitable method for use with the human dental pulp. Although the Fast-Prep®-24 machine provided the greatest concentration of undegraded RNA_{total} (ng/ μ l), the cost and availability of equipment were limiting factors in this study. RNA_{total} with a similar concentration (ng/ μ l) and marginally higher level of degradation was obtained when tissue was homogenised in a glass Dounce tissue homogeniser on ice. Concentrations achieved with this method were thought to be lower compared to the Fast-Prep®-24 machine as the manual grinding of the tissue did not break up the sample as completely as the Fast-Prep®-24 machine, and collagenous fibres remained. Although resulting in a marginal loss of RNA_{total} mass (ng), this method was chosen as the most appropriate method for human dental pulp homogenisation as the RNA_{total} integrity remained in tact.

Although three methods for RNA isolation were examined using MG-63 osteosarcoma cells as human dental pulps samples were unavailable at the time of experimentation

(data not shown); phenol:chloroform isolation, a biphasic liquid-liquid extraction protocol, in combination with DNase treatment was discounted immediately as high levels of organic compound contamination were present and DNase treatment was shown to reduce the extracted yield of RNA_{total} (ng) when quantified by spectrophotometry. High levels of organic compound contamination were thought to be due to manual error occurring during the separation of the upper aqueous phase from the lower phase; or from inefficient ethanol precipitation which purifies and concentrates RNA from an aqueous solution.

The RNEasy Mini Kit, a well-known commercial product limiting the incidence of manual error by using a filtered-column system, was used in combination with DNase treatment and although the organic compound concentrations (ng/μl) were lower in comparison to the phenol:chloroform extraction protocol when quantified by spectrophotometry; RNA_{total} yields (ng) remained lower than expected and this was thought to be due to the separate stage of DNase treatment. This separate stage of DNase treatment removes genomic DNA from RNA_{total} samples, reducing the overall yield (ng) when samples are quantified by spectrophotometry. In contradiction to our hypothesis of DNase treatment reducing overall RNA_{total} yield (ng) by negative effect, the larger yields (ng) obtained from isolation of RNA_{total} without DNase treatment were in fact samples contaminated with genomic DNA that could not be detected due to the quantification protocol used at this point in the study. Following Bioanalysis, it was confirmed that DNase treatment should be excluded from the study, as samples treated with DNase contained higher levels of genomic DNA contamination than those that had not been treated (section **3.4.2.1**). This was contrary to our expected hypothesis as DNase treatment should remove genomic contamination and we have no reasonable proposal by which to substantiate this finding.

The RNEasy Mini Kit without DNase treatment was compared against a modified phenol:chloroform protocol used in combination with the RNEasy Mini Kit, under the direction of Dr N. Silver, and this was found to produce greater yields (ng) of RNA_{total} with low levels of organic compound degradation when assessed by Bioanalysis (section 3.4.2.1). As this method produced the largest yield (ng) of RNA_{total} with the lowest level of degradation, this method is suggested as the most appropriate for isolating RNA_{total} from the human dental pulp in our experimental system.

3.5.2.2 *Assessment of RNA Integrity*

Bioanalysis was performed on a combination of tissue homogenisation and RNA isolation approaches and each was assigned an RNA Integrity Number (RIN). RIN values range from 10 to 1 showing a scale of increased gradual degradation from fully intact RNA to totally degraded RNA, respectively. This is depicted in the electrophoretic traces as a shift from two clear tall peaks, representing the 18S and 28S ribosomal RNA subunits, to shorter, indistinct fragment sizes; and eventually a single peak next to the axes origin, representative of complete degradation (Schroeder *et al.* 2006). Samples assessed from all combinations of tissue homogenisation and RNA isolation showed shorter peaks throughout the trace, indicating some level of sample degradation in each homogenisation-isolation approach. The most degraded sample, isolated in a mortar and pestle, was assigned an RIN of 2.9 and showed a large peak, representative of genomic DNA contamination (Caruana 2004) and low peak at the 28S subunit. The height of the 28S peak is an important indicator of RNA degradation, as during degradation this peak will disappear faster than the 18S peak (Schroeder *et al.* 2006). A RIN was not assigned to the electrophoretic trace from the most intact sample, isolated in the Fast-Prep machine; despite there being a strong peak at the 18S subunit and very few shorter fragments between ribosomal subunit peaks and to the left of the

18S peak. Had the assessment been able to be performed in duplicate, it is the opinion of the researcher that a high RIN would have been assigned to this experimental approach. The approach utilising the modified phenol:chloroform isolation technique and the Dounce tissue homogeniser was selected for further use in this study as it was assigned a RIN of 5.4 and showed limited genomic DNA contamination in comparison to the approach used by isolating the RNA while passing the sample through a needle. According to the literature, there is no current level of sample degradation that would render a sample as unusable in RT-qPCR analysis, although it has been established that the optimal template for RT-qPCR will have an RIN of 8 or more; whilst partly degraded RNA_{total} with an assigned RIN in the range of 8 – 5 may result in suboptimal RT-qPCR gene expression results (Fleige *et al.* 2006, Fleige and Pfaffl 2006, Gallego *et al.* 2014). In order to correct for RNA integrity, it has been suggested that primers for the GOIs are designed across an internal region of the gene and that the RT-qPCR product be no more than 200 bp in length (Fleige *et al.* 2006, Fleige and Pfaffl 2006). As our combined homogenisation and isolation approach produced RNA_{total} of partly degraded integrity, we corrected for this during the primer design stage of the experiment.

3.5.2.3 *Optimisation of Protocols for Quantification of RNA Yields*

Conventional methods of sample assessment are often not sensitive or specific enough to identify variations in the quantity and quality of isolated RNA_{total} (Bustin *et al.* 2005, Imbeaud *et al.* 2005). We have demonstrated that there is a statistically significant difference ($p < 0.05$) in the amount (ng) of RNA_{total} considered to be attainable when quantified by the fluorescence-based ‘Quant-iT™ Ribogreen® RNA Assay Kit’ when compared with quantification by NanoDrop™ UV spectrophotometry. These differences may be attributed to the sensitivity levels of each assay. At concentrations

lower than 100 ng/μl, UV spectrophotometry has proven to be unreliable as proteins, genomic DNA and free nucleotide levels are measured at the 260 nm wavelength alongside RNA (Bustin 2000, Fleige and Pfaffl 2006). However, at concentrations of 100 ng/μl or more, UV spectrophotometry presents similar yields to the Ribogreen® assay (Bustin 2002). As our samples produced a mean (\pm SEM) yield of 26.89 \pm 1.11 ng/μl and the Ribogreen® assay is able to detect as little as 1 pg/μl RNA, we can conclude that the Ribogreen® assay is the most sensitive technique for quantifying RNA_{total} isolated from human dental pulp samples (Fleige and Pfaffl 2006, Jones *et al.* 1998).

3.5.3 Optimisation of Protocols for RT-PCR and RT-qPCR

3.5.3.1 Primer Design

Following quantification, RT-qPCR protocols were optimised by reducing variability at the stages of primer design, standardising RNA_{total} inputs (ng) and reverse-transcription. Poor RNA integrity has been shown to have significant effects on detected expression levels when amplicon lengths exceed 200 bp (Fleige and Pfaffl 2006). This is because longer amplicons are not well suited to endure the prolonged exposure to high temperatures in the RT-qPCR reactions, which may result in degradation of the amplicon. Shorter amplicons would separate efficiently in the denaturation stage of the PCR, primers would then compete more effectively for their binding sites during the annealing stage and the extension stage could be shortened to as little as 15 sec; which would also minimise the chances of amplifying genomic DNA (Bustin 2000, Jeffreys *et al.* 1988). By designing amplicons within the range of 73 bp – 203 bp, the influence of RNA integrity on the reaction was reduced.

Whilst Primer-BLAST analysis was carried out for all primers, thus ensuring primer specificity to the target GOI; some of the primers designed for RT-PCR did not produce

gene-specific peaks in the dissociation/melt curve analysis in RT-qPCR (data not shown). Primers for RT-PCR were therefore not suitable for use with the highly specific RT-qPCR technique and new primers had to be designed using Primer-BLAST in conjunction with Primer Express® Software v 2.0. By performing RT-PCR, followed by gel electrophoresis, we were able to determine that the GOIs were expressed in both MG-63 osteosarcoma cells and in the human dental pulp. It is important to note that while RT-PCR is a good method for determining gene expression in target samples and potential experimental calibrators, and that this is a fundamental step to the inception of any experiment where gene expression has not been previously reported in the target sample; if RT-qPCR is to be the predominant method for gene expression analysis, it may be better to optimise the experimental conditions, primers and reagents using RT-qPCR from the onset. Although RT-qPCR is more expensive, optimisation of experimental conditions within this protocol may save a lot of time and prevent the unnecessary purchase of reagents and primer sets which may be efficient in RT-PCR but are unsuitable for RT-qPCR analysis.

3.5.3.2 *Standardising Amount of RNA Input and Reaction Reagents*

Standardising the amount (ng) of RNA input into each reaction ensures that changes observed in gene expression are not artefacts of cell state, as RNA isolated from complex tissues is recognised to contain a wide range of cell types at varying stages of differentiation (Bustin *et al.* 2005, Vandesompele *et al.* 2002). RNA_{total} input was standardised at 10 ng per reaction.

Reverse-transcription yields can vary by up to 100-fold according to which reverse-transcriptase is used and this variation is reflected in the genes that are subsequently analysed (Stahlberg *et al.* 2004). In the reverse-transcription of RNA_{total} to cDNA in the MG-63 osteosarcoma cells for RT-PCR, an MMLV-reverse transcriptase enzyme was

used. This enzyme is considered to be the best enzyme to use when amplification of full-length cDNA molecules is required (Bustin 2000). The details of the reverse-transcriptase enzymes used in the iScript one-step kit and in the RNA Amplification System were withheld by the manufacturers. Therefore, no conclusion could be made as to which reverse-transcriptase enzyme should be utilised with preference in this study.

The primers for reverse-transcription are also known to have a significant effect on the cDNA produced and the subsequent down-stream gene expression analysis, as each priming technique offers advantages and disadvantages. The iScript one-step RT-qPCR kit utilises gene-specific primers. Whilst this method of reverse-transcription produces highly specific cDNA for the GOI, which is then amplified directly in PCR; the use of gene-specific primers only allows for a select number of genes to be analysed from the samples in relation to the amount of RNA_{total} initially isolated (Bustin 2000, Bustin *et al.* 2005, Lekanne Deprez *et al.* 2002). This is an additional limiting factor that should be excluded when samples are of a rare, clinical origin. The MMLV-RT method for MG-63 osteosarcoma cells uses random primers, whilst the RNA Amplification System uses a combination of random and Oligo-dT primers. Random primers will anneal to the RNA at multiple points along the strand, resulting in a number of cDNA strands of varying lengths. The advantage of this method is that a large amount of cDNA is produced in the reverse-transcription reaction, thus allowing the quantification of a wide range of genes; however the sections of the RNA containing the GOIs may not be transcribed proportionately leading to an over- or under- estimate of mRNA copy number present in the sample (Bustin 2000, Bustin *et al.* 2005, Zhang and Byrne 1999).

It is anticipated that any potential alteration of the real expression profile of mRNA, as a result of reverse-transcription with random primers, will occur for all genes expressed within the tissue. Thus, by normalising to an internal reference gene, thought to be expressed by all cell types, at a maintained level of expression; these over- or under-

estimates of the mRNA copy are negated (Resuehr and Spiess 2003). Oligo-dT primers bind to the poly(A)-tail of 3'-end of the RNA strand and will prime only mRNA sequences unlike random primers which also transcribe rRNA. The disadvantage to this type of primer is that degraded RNA will be difficult to transcribe and if samples are reverse-transcribed, there is potential for biased reverse-transcription of the 3'-end (Bustin *et al.* 2005, Resuehr and Spiess 2003). By combining random priming with Oligo-dT priming, the RNA Amplification System maximises the experimental potential of a sample by producing large volumes of cDNA representative of the mRNA present in the original sample from both the 5' to the 3'- ends of the RNA strands. As the mean yield of cDNA following amplification were in the magnitude of 8,000 ng per reaction, it is assumed that any potential degradation of the isolated sample RNA_{total}, which would affect reverse-transcription with the Oligo-dT primers, was counterbalanced by the random primers in the reaction.

By modifying the amount of reagents and RNA_{total} input into the reverse-transcription of MG-63 osteosarcoma cells, we showed the importance of experimental optimisation in order to increase the experimental potential of our samples.

3.5.3.3 One-Step RT-qPCR vs RNA Amplification : Getting the most out of samples with small RNA_{total} yields

The one-step RT-qPCR system was used for all samples in this chapter, reducing the risk of introducing manual error in separate reverse-transcription and RT-qPCR steps (Nolan *et al.* 2006). Although use of a one-step system is beneficial, these systems are limited by the amount (ng) of RNA isolated from the samples; i.e. the less RNA isolated, the fewer number of genes can be analysed. We have shown that reverse-transcription using an RNA Amplification System eliminates the experimental limitations of RNA_{total} yield, maximising the potential of individual pulp samples and

eradicating the need for sample pooling. In order to analyse a larger panel of genes in the same samples, the RNA Amplification System was used throughout the remaining chapters of this thesis and we are of the opinion that this system should be utilised by groups analysing gene expression in rare, clinical samples where tissue mass and isolated RNA is limited. It is critically important to note that RT-qPCR results are only directly comparable when the “same priming strategies and reaction conditions” are used (Bustin *et al.* 2005, Resuehr and Spiess 2003), therefore the GOIs analysed within this chapter were assessed again alongside the extended panel of target genes using the RNA Amplification System and a separate RT-qPCR step.

3.5.4 Optimising Protocols for Normalising RT-qPCR Data

In order to reduce sample-to-sample and run-to-run variability by removing sampling noise such as a difference in RNA_{total} concentration or quality, RT-qPCR data is normalised against a stable internal control. It is known that variation in reference gene expression introduces error and obscures real changes in expression of target genes (Bustin 2000, Bustin 2002, Bustin *et al.* 2005, Toegel *et al.* 2007); and it is therefore fundamental that an appropriate gene, which is stably expressed and is not co-regulated under experimental conditions, is carefully selected for each experimental system. A panel of reference genes was selected for stability assessment from three published lists, compiled from a range of tissue types in the human (Eisenberg and Levanon 2003, Hsiao *et al.* 2001, Warrington *et al.* 2000), and an additional list of supplementary microarray data from the human dental pulp (Paakkonen *et al.* 2005). One-step RT-qPCR was performed on RNA_{total} isolated from samples treated with an orthodontic force for a period of two weeks and on contralateral intra-patient controls.

3.5.4.1 Normalising Against RNA Input Amount

Initially, C_q values were compared directly to assess changes in mRNA expression between the control and treated pulps; and normalised against the amount (ng) of total cellular RNA, a mixture of microRNA, mRNA (~ 1 – 5 %), tRNA (~ 15 – 20 %) and rRNA (~ 80 – 85 %), input into each reaction. It is assumed that equal amounts (ng) of RNA contain the same mRNA and rRNA fractions; that the ratio of the fractions will not change under the experimental system; and that there is little variability in total RNA content within the same tissue between individuals (Bustin 2000, Bustin 2002). However, it has previously been shown that the mRNA:rRNA ratio has a tendency to fluctuate under some experimental systems and can become imbalanced under physiological conditions (Hansen *et al.* 2001, Schmittgen and Zakrajsek 2000, Solanas *et al.* 2001, Spanakis 1993, Tricarico *et al.* 2002). From **Figure 7** we can see that changes in the mRNA and rRNA fractions under this experimental system are minimal as 5 of the candidate reference genes analysed appear to have similar expression distributions between the control and treated groups and between the genes themselves and that no significant differences have been found ($p > 0.05$) (**Table 22**).

3.5.4.2 Assessing the Variance in Reference Gene Expression

The variance of C_q values was then assessed by examining the mean and SD for each reference gene. According to Dheda *et al.* (2004) a suitable reference gene should display a standard deviation of less than 2-fold (based on a logarithmic scale) or less than 1 C_q value change relative to the mean, assuming 100% reaction efficiency. Our study identified that, according to the variance of C_q values (data not shown), candidate genes should be ranked as follows from most to least stable: (1) HPRT1; (2) UBC; (3) GAPDH; (4) RPL13A; (5) ACTB; (6) YWHAZ; (7) PPIA. As normalising against total RNA and analysing C_q variance does not take reaction efficiency or the relationships of

expression between each reference gene into consideration, further statistical analysis was performed to substantiate our findings.

3.5.4.3 Identifying a Stable Reference Gene: BestKeeper Analysis

The Excel-based tool, *BestKeeper*© assumes that C_q values are normally distributed in most cases and linearises data by a logarithmic transformation to the base of two (Pfaffl *et al.* 2004). Following linearisation, reaction efficiency is corrected for and a pair-wise correlation analysis is utilised, assuming samples have equal variance, to calculate the stability of each reference gene and assess how similarly each gene is expressed. HPRT1 and PPIA were shown to have different coefficients of variation in relation to the rest of the panel as they were expressed at much lower levels than the other GOIs (**Table 23**); and were subsequently excluded from further analysis as it is recommended that reference genes should be expressed at similar levels as other reference genes and the GOIs (Dheda *et al.* 2004, Toegel *et al.* 2007). The remaining genes were compared and the Pearson correlation coefficient was calculated along with the probability that correlation is significant (p-value). As all five genes were found to be significantly correlated (**Table 24**), the data was combined and the geometric mean was calculated to provide a normalisation factor referred to as the *BestKeeper*© index. Correlation between individual reference genes and the *BestKeeper*© index was assessed by regression analysis and all five genes were found to be significantly ($p < 0.05$) correlated to the index (**Table 24**). As care was taken to minimise co-regulation of the candidates, by selecting genes from different functional groups, we can assume that results produced by *BestKeeper*© are an accurate evaluation of mRNA expression in these samples, and that these reference genes are similarly expressed under our experimental system.

3.5.4.4 Identifying a Stable Reference Gene: geNorm and NormFinder Analysis

In contrast to *BestKeeper*®, which identifies correlation and therefore similarity in candidate gene expression, the geNorm and NormFinder algorithms, embedded in GenEx software version 4.3.5, were used to identify differences in candidate gene expression. In their original study, Vandesompele *et al.* (2002) identified that the Pearson and Spearman correlation coefficients were unsuitable for statistical analysis of a ratio created from raw C_q values, as the data range and outlying values were found to have a profound effect on the correlation coefficient. For this reason logarithmic transformation of raw data was carried out in order to symmetrically distribute the data around zero (Vandesompele *et al.* 2002). This finding highlights the error of normalising against total RNA input (ng) and assessing gene stability by analysing C_q variance; as in these two methods, logarithmic data is compared directly using parametric tests such as the paired Student's t-Test where equal variances are assumed. This finding emphasises the importance of further statistical analyses by software such as *BestKeeper*®, geNorm and NormFinder, which correct for the assumption of data distribution and for reaction efficiency. Using the geNorm function, reference genes were compared pair-wise, one gene against all other genes, in order to calculate the standard deviation or M-value (gene expression stability) of each ratio. Genes were ranked by implementing a step-wise exclusion process, removing the gene with the highest M-value and the highest degree of instability (**Figure 8**). This study has shown RPL13A and UBC to be the most stable pair under this system and corroborates the *BestKeeper*® findings.

Andersen *et al.* (2004) proposed that all genes, reference or otherwise, will show variation in expression to an extent under any experimental system; and suggested that in order to normalise accurately, it is fundamental to assess variation of the reference

genes in the experimental subgroups in addition to overall expression variation (Andersen *et al.* 2004). Using the NormFinder function to assess overall expression variation, genes were ranked from least to most stable (**Table 25, Figure 9**). Disparities seen between the geNorm and NormFinder analysis, amongst the least stable genes, can be accounted for by the differences between the model-based (Andersen 2004) and the pair-wise comparison (Vandesompele *et al.* 2002) approaches. While geNorm attempts to identify a pair of reference genes with similar expression profiles and low intra-group variation; NormFinder attempts to identify a single gene with the least variation between the overall expression variation and that occurring between the subgroups.

3.5.4.5 Reference Genes: The More the Merrier

Vandesompele *et al.* (2002) have further suggested that normalisation can only be performed accurately when using the geometric mean of multiple reference genes but have outlined a number of limitations to this method including the wasteful use of multiple reference genes when there are limited resources and amounts of RNA available (Dheda *et al.* 2004, Vandesompele *et al.* 2002, Vandesompele *et al.* 2009.). They conclude that normalising against a single gene, whose stability has been assessed and verified, is a reasonable alternative when limitations apply. As the one-step RT-qPCR kit used in this chapter limited us to analysing only a small panel of genes in our samples, we compared normalising C_q values against UBC alone and against the geometric mean of the two most stable genes RPL13A and UBC. This was done in order to illustrate the importance of normalising gene expression data against a verified combination of reference genes and to show that despite the limitations of sample yield, using the appropriate reference gene combination is more important than analysing a wider panel of genes.

3.5.5 Relative Expression Analysis of RT-qPCR Data from Orthodontically Treated Pulps

To assess the effects of orthodontic force application on pulp microvasculature, RT-qPCR was performed with primers specific to four angiogenic growth factors and C_q values were input into Microsoft Excel ® spreadsheet software and the REST-2009© software package (Pfaffl *et al.* 2002).

3.5.5.1 ΔC_t and $\Delta\Delta C_t$

It was shown that assessment by the ΔC_t and $\Delta\Delta C_t$ methods resulted in the down-regulation of mRNA expression of all target genes as a result of orthodontic force application. This result opposes the hypothesis that the application of an orthodontic force will initiate the increase of mRNA expression and therefore protein expression of angiogenic growth factors in the human dental pulp. While there was no significant difference between the two methods, it should be noted that the ΔC_t method compares all samples us unrelated, individual cases whilst the $\Delta\Delta C_t$ method considers the change in expression directly within pairs of treated and control samples (Livak and Schmittgen 2001, Schmittgen and Livak 2008). In the ΔC_t method, target gene expression is normalised against reference gene expression in the sample and this normalised value is log transformed. The mean normalised expression of the log transformed data is then calculated for the control and treated samples respectively. The treated samples are then compared against the control samples in order to determine the fold change between the samples, the negative reciprocal of this fold-change is then calculated to determine the fold-change between the samples due to treatment (Livak and Schmittgen 2001, Schmittgen and Livak 2008). A negative value indicates a down-regulation of mRNA expression whilst a positive value would indicate that treatment induces an increase or up-regulation of mRNA expression. The $\Delta\Delta C_t$ method

does not compare samples individually but rather assumes that all changes in the control will be directly related to the treated group as control and treated samples are paired from the same individual. Initially, the mean C_q is determined for the GOI and the normaliser in both the control and treated groups. The mean target gene expression is then normalised against the mean reference gene expression in the control and treated groups, respectively. The treated group is then compared against the control group to determine the Delta-Delta C_t value. This is then log transformed and the negative reciprocal of the log-transformed value is considered to be the fold-change between the two groups due to treatment (Livak and Schmittgen 2001, Schmittgen and Livak 2008). As with the Delta C_t method, a negative value indicates down-regulation of mRNA expression and a positive value indicates up-regulation. The use of the Delta C_t method is of significance in samples isolated from a large population where no direct comparison can be made, e.g. in cancer studies where patients either do or do not have cancer; there is no case in which a non-biased control can be obtained from a patient that is expressing a cancerous phenotype and therefore an external control has to be used. This method was used in this study to determine whether or not there was a significant difference between the samples treated as individual data-points and those treated as control-treated contralateral pairs. There was no significant difference between the two methods, although it should be noted that in every gene with the exception of PDGFA, down-regulation of mRNA expression as a result of treatment was lower in samples compared as contralateral pairs. This is due to the assumption that any changes between the samples are directly comparable and are a result of treatment. By using the Delta-Delta C_t method we are able to see direct responses to treatment and are able to limit sample-to-sample variation and false interpretation of data.

Although there was no significant difference observed when comparing gene expression by normalising against: (1) the single gene, UBC and (2) the geometric mean of

RPL13A and UBC, previously found to be the most stably expressed pair of reference genes; it was observed that there was less down-regulation of mRNA expression in those genes normalised against the geometric mean of the two reference genes. This observation is thought to be due to the additional correction made by incorporating another reference gene, restricting the error introduced by a loose normalisation approach and regulating for possible co-regulation of the reference genes and target genes under the experimental conditions.

3.5.5.2 *Correcting for Reaction Efficiency using the Relative Expression Software Tool*

Previously, it has been shown that amplification efficiency plays a fundamental role in the determination of the threshold levels and points of quantification in each reaction (Pfaffl *et al.* 2004, Vandesompele *et al.* 2009); and that a difference in RT-qPCR efficiency will produce false expression ratios (Freeman *et al.* 1999, Peirson *et al.* 2003, Pfaffl *et al.* 2002). It is therefore crucial that efficiency is corrected for prior to determination of relative gene expression. The Delta C_t and Delta-Delta C_t methods do not correct for reaction efficiency and this may be the reason that an expected up-regulation of mRNA expression was determined to be a down-regulation in mRNA expression as a result of orthodontic treatment prior to extraction.

REST-2009© software calculates relative gene expression using an equation derived from the Delta-Delta C_t equation outlined by Livak and Schmittgen (2001) and a modification of Pfaffl *et al.*'s (2001) efficiency corrected model: Relative Expression

$$\text{Ratio} = \frac{[E_{\text{GOI}}^{(\text{Mean Control} - \text{Mean Treated})}]}{[E_{\text{REFERENCE GENE}}^{(\text{Mean Control} - \text{Mean Treated})}]}$$

Subsequent significance testing is carried out by the Pair-Wise Fixed Reallocation Randomisation Test© (Pfaffl *et al.* 2002) and by a hypothesis test using bootstrapping

techniques (<http://rest-2008.gene-quantification.info/>); and expression ratios are represented in box-whisker plots to show the distribution of the data.

3.5.5.3 Normalising RT-qPCR Data against a Single Reference Gene Compared to a Pair of Reference Genes

Our study showed that when normalising against UBC, there were no significant changes in the mRNA expression of FGF2, PDGFA, TGFB1 and VEGFA in dental pulps removed from teeth that had been treated with an orthodontic force for two weeks prior to extraction (**Figure 11**). However, when C_q values were normalised against the geometric mean of RPL13A and UBC, the results showed that FGF2 was significantly up-regulated in the treated samples relative to the controls. This finding highlights the argument raised by Vandesompele *et al.* (2002) where a normalisation factor created from the geometric mean of multiple reference genes is more appropriate for normalising gene expression levels than a single candidate gene (Vandesompele *et al.* 2002).

Although there was an expectation of identifying an up-regulation of mRNA for all four of the angiogenesis-associated growth factors, as the morphological phenotype of increased vasculature was seen after a few days in the co-culture system used by Derringer *et al.* (2003), it is important to note that angiogenesis like any other biological process is a complex mechanism (Folkman and Shing 1992) and there may be a delay between the transcription of mRNA and translation to protein. From this we predict that alterations in mRNA expression are more likely to occur in the initial response to force application. In order to assess the effects of orthodontic treatment on pulpal microvasculature at the transcriptional stage we propose that the expression patterns of a larger panel of angiogenesis-associated genes is evaluated at a number of earlier time-points during orthodontic treatment.

3.5.5.4 *Normalising RT-qPCR Data against Reference Genes Identified as Unstable under our Experimental Conditions*

The effect of normalising with individual generic reference genes was subsequently assessed (**Figure 13**). In this experiment it was shown that a number of genes can be interpreted to be significantly ($p < 0.05$) up-regulated according to the influence of a variable reference gene. These findings confirm our initial statement that normalising against a reference gene, which has not been verified for stability under a given experimental condition may result in erroneous interpretation of expression data. For the purpose of this study, all further gene expression data was normalised against the geometric mean expression of the reference genes RPL13A and UBC.

As PCR efficiency has been demonstrated to be affected by the tissue type analysed and the tissue homogenisation and isolation of RNA_{total} protocols used; and has also been shown to have a down-stream effect on the generation of false gene expression ratios, its correction cannot be ignored. (Booth *et al.* 2010, Fleige *et al.* 2006, Pfaffl 2001). We therefore propose that the Delta C_t and Delta-Delta C_t methods be avoided in future studies and that every measure be taken to increase experimental efficiency to 100 %. In order to minimise any additional error in the remaining experimental chapters, where the panel of target genes was expanded to obtain a better understanding of the angiogenic response in human dental pulps as a response to orthodontic force application at varying time-points of initial treatment; primers for RT-qPCR were designed by the company Primer Design Ltd., Southampton, UK which guaranteed a reaction efficiency of 100 % when used in conjunction with their Precision Mastermix with SYBR Green.

3.6 Conclusion

The human dental pulp has previously been shown to demonstrate increased microvasculature, in co-culture, following orthodontic force application and is an interesting model for the study of angiogenic growth factors. Previous studies assessing gene expression in this tissue have used reference genes for normalisation that have not been validated for expression stability (Dissanayaka *et al.* 2015, Gronthos *et al.* 2002, Liu *et al.* 2007, McLachlan *et al.* 2005). In order to ensure that any small changes in gene expression were measured accurately, a number of techniques were evaluated and optimised to eliminate the introduction of manual and experimental error. We identified that:

1. RNA stabilisation solutions are more appropriate for sample collection under clinical conditions than snap-freezing samples in liquid nitrogen.
2. The most commonly used technique for RNA quantification, UV spectrophotometry, is not sensitive enough to accurately measure the small yields of RNA isolated from this tissue.
3. Elimination of the experimental error, produced in an additional reverse-transcription step, by using a one-step RT-qPCR kit introduces limitations derived from the amount (ng) of total RNA isolated from a sample.
4. Under non-limiting conditions where an RNA amplification system is used to produce large volumes of cDNA, the geometric mean of RPL13A and UBC is the most appropriate normalising factor under our unique experimental conditions.
5. Under limiting conditions, where an RNA amplification system is unavailable, UBC alone could be used for normalising gene expression data as it is the single most stable reference gene under our experimental conditions, although this is unadvisable as spurious results may be derived.

6. Reaction efficiency is critical to the accurate determination of relative mRNA expression.
7. Protocols for determining fold-change such as the Livak methods (Livak and Schmittgen 2001, Schmittgen and Livak 2008), which do not account for efficiency correction, should only be used where efficiency is calculated to be 100% through the use of internal standard curves.

This experimental chapter outlines the call for optimisation of experimental protocols prior to data acquisition and highlights the specific nature of reference genes in relation to a particular experimental model; both must be evaluated thoroughly and methodically in order to prevent erroneous interpretations of relative gene expression ratios.

Chapter IV

Effects of Orthodontic Force Application on mRNA and Protein Expression of Angiogenic Growth Factors in Human Dental Pulp

4.1 Introduction

The human dental pulp is a highly innervated, loose, vasculature tissue enclosed within an unyielding case of dentine, cementum and enamel (Grando Mattuella *et al.* 2007, Ramazanzadeh *et al.* 2009); and is a known source of regulatory cytokines and growth factors (Artese *et al.* 2002, Paakkonen *et al.* 2005, Roberts-Clark and Smith 2000, Shimabukuro *et al.* 2009, Smith 2002, Tete *et al.* 2008, Tran-Hung *et al.* 2006, Zhong *et al.* 2012).

It is recognised that alteration to the pulp's unique and noncompliant environment by microbes; trauma or iatrogenic therapies induces dramatic and often irreversible changes in the tissue, such as pulpitis or necrosis (Bjorndal and Ricucci 2014, Heyeraas and Berggreen 1999, Nagata *et al.* 2014, Rodd *et al.* 2005, , Schmalz 2014, Vianna *et al.* 2007, Yu *et al.* 2007)

In particular, routine orthodontic treatment has been shown to be associated with a number of morphological and physiological changes of the pulp including a reduction in pulpal blood flow (Sano *et al.* 2002, Yamaguchi 2007) alteration of the microvasculature (Santamaria *et al.* 2006, Yamaguchi 2007), congestion of the vasculature (Lazzaretti *et al.* 2014, Mostafa *et al.* 1991) and increased secretion of diffusible angiogenic growth factors (Derringer *et al.* 1996, Derringer and Linden 2003, Derringer and Linden 2004, Derringer and Linden 2007). However, the full impact of orthodontic treatment on the human dental pulp has yet to be comprehensively characterised, particularly from the perspective of the angiogenic response to treatment. Angiogenesis is the process of new vessel formation from a pre-existing capillary plexus (Brown *et al.* 1992, Li J, Yano *et al.* 2003, Zhang *et al.* 2003) and is known to occur in the adult as a: (a) result of localised hypoxia, (b) reaction to tissue damage or (c) an imbalance of regulatory cytokines (Carmeliet 2000, Risau 1996, Risau 1997).

This complex signalling cascade has been shown to occur by one or two processes concurrently: sprouting angiogenesis (Carmeliet 2000, Folkman and D'Amore 1996) and intussusceptive microvascular growth (Burri *et al.* 2004, Djonov *et al.* 2000) and is tightly regulated by a large number of cytokines, chemokines and growth factors.

Some of the growth factors, cytokines and chemokines known to be involved in the angiogenic signalling cascades include: ANGPT1 (Aplin *et al.* 2006, Brindle *et al.* 2006); FGF2 (Li *et al.* 2003, Presta *et al.* 2005); HIF1A (Hirota and Semenza 2006, Ke and Costa 2006, Pugh and Ratcliffe 2003); interleukin 8 (IL8) (Li *et al.* 2003, Ning *et al.* 2011); ITGAV (Bader *et al.* 1998, Weis and Cheresh 2011); ITGB3 (Hodivala-Dilke *et al.* 1999, Serini *et al.* 2006); KDR (Hicklin and Ellis 2005, Hoeben *et al.* 2004, Terman *et al.* 1992); MMP2 (Bellafiore *et al.* 2013, Rundhaug 2005); NOS2 (Kroll and Waltenberger 1998, Luo and Chen 2005); PDGFA (Palomero *et al.* 2014, Shikada *et al.* 2005, Tran-Hung *et al.* 2008); platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog) (PDGFB) (Li *et al.* 2010, Ohlsson *et al.* 1999, Tran-Hung *et al.* 2008); roundabout homolog 4, magic roundabout (*Drosophila*) (ROBO4) (Jones *et al.* 2008, Yadav and Narayan 2014); SELE (Koch *et al.* 1995, Nishiwaki *et al.* 2007); TEK tyrosine kinase, endothelial (TEK) (Lin *et al.* 1997, Singh *et al.* 2011); TGFB1 (Carmeliet 2000, Nakagawa *et al.* 2004); TGFBR1 (Nakagawa *et al.* 2004, Sankar *et al.* 1996) and VEGFA (Folkman and D'Amore 1996, Gerhardt *et al.* 2003, Hicklin and Ellis 2005, Hoeben *et al.* 2004).

Several of these cytokines and growth factors were previously shown to be released from the human dental pulp in response to orthodontic force application (Derringer *et al.* 1996, Derringer and Linden 2003, Derringer and Linden 2004) and their mRNA expression levels have been investigated in **Chapter 3** following a period of two weeks of routine orthodontic treatment; however, the expression of more growth factors and cytokines involved in the angiogenic signalling cascades has not yet been considered.

Therefore, in order to gain a greater perspective of the angiogenic response of the human dental pulp to the early stages of routine orthodontic treatment, our initial experimental time-point was expanded from 2 weeks to three early time-points: 1 day, 3 days and 14 days (2 weeks); and the panel of angiogenesis-specific genes was expanded from four angiogenic growth factors to a panel of seventeen GOIs.

4.2 Aims

1. Expand the panel of angiogenic growth factors previously assessed in order to more fully comprehend the angiogenic response of the human dental pulp to orthodontic force application.
2. Expand the treatment time-point of orthodontic force application previously investigated from 2 weeks to: 1 day, 3 days and 14 days, respectively; in order to characterise the human dental pulp's angiogenic response to the early stages of routine orthodontic treatment.
3. Identify whether proteins previously seen to be secreted by pulps in response to orthodontic force application are transcribed earlier than our original anticipated time-point of two weeks force application.
4. Determine relative changes in mRNA and protein expression in orthodontically treated pulps compared with untreated controls; linking these changes with known patterns in the angiogenic signalling cascades.

4.3 Materials and Methods

MATERIALS

4.3.1 Human Dental Pulps

Following informed parental and patient consent, human dental pulps were obtained from healthy first and second premolar teeth extracted from volunteers aged between 12 – 16 years. All patients included in the study required extraction of a minimum of 2 paired, contralateral premolar teeth in conjunction with fixed appliance orthodontic treatment. Ethical approval was granted by the Research Ethics Committee at Guy's Hospital and King's College Hospital.

METHODS

4.3.2 Orthodontic Force Application and Dental Pulp Isolation

In this experimental chapter, pulps were isolated from 30 pairs of 'control' and contralateral 'treated' teeth, in order to analyse the relative mRNA expression of angiogenesis-specific genes in human dental pulps at three early time-points in routine orthodontic treatment: 1 day, 3 days and 14 days. 10 contralateral pairs, 2 pulps per patient, were randomly assigned to each treatment time-point group prior to orthodontic force application.

A further 15 contralateral pairs of 'control' and 'treated' teeth, 2 pulps per patient, were collected in order to assess protein expression following orthodontic force application. For each of the three time-points analysed, 5 contralateral pairs were randomly assigned to a group prior to orthodontic force application.

In every patient, the oral cavity was divided along the mid-sagittal plane in order to assign a 'control' sample that would forego orthodontic force application; and a

contralateral ‘treated’ sample which would experience a force in the range of 0.5 – 1 N for the duration of treatment. Fixed appliance straight wire orthodontic brackets (Roth 0.022 inch, 3M Unitek, California, USA) were direct bonded (Transbond, 3M Unitek, California, USA) to the upper and/or lower teeth from second premolar to second premolar, except the assigned ‘control’ teeth. Orthodontic bands were cemented (Ketac, 3M ESPE, Seefeld, Germany) onto all first permanent molar teeth. Test tooth position was carefully assessed and brackets were bonded in a position (offset if necessary) so that the archwires (0.016 inch nickel-titanium, TP Orthodontics Inc., Leeds, UK) placed gave the required force of 0.5 – 1 N in a mesial and extrusive direction (Derringer *et al.* 1996).

Following the required period of force application (1 day, 3 days or 14 days respectively), brackets were removed from the premolar teeth assigned as ‘treated’ teeth. The ‘control’ and contralateral ‘treated’ premolar teeth were extracted under local anaesthesia, placed in individual tubes containing 20 ml of cold sterile saline solution, and sectioned immediately with a high-speed water-cooled diamond bur through the buccolingual aspect. Dental pulps with a mean mass of ± 0.05 g ($n = 5$) were removed from the pulp chamber with sterile blunt instruments. Isolated pulps to be used for proteomic analysis were individually placed in a sterile 1.5 ml centrifuge tube and snap-frozen in liquid nitrogen before transferring to -70°C for permanent storage. Isolated pulps to be used for transcriptomic analysis were immersed separately in 500 μl RNeasy Lysis Buffer (Cat. No. AM7021) (Ambion Inc., Warrington, UK), an RNA preservation solution, kept at 4°C for 8 hr and transferred to -20°C for permanent storage.

All orthodontic procedures and dental pulp isolations, following tooth extraction, were performed by Dr K. A. Derringer, Dental Institute, King’s College London at Guy’s Hospital and King’s College Hospital.

4.3.3 RNA Isolation

Pulp samples stored in 500 µl RNALater™ were removed from solution and ground individually in a sterile, autoclaved 7 ml glass Dounce tissue homogeniser (Wheaton Science Products from VWR, Poole, UK) containing 600 µl of the RNA stabilisation solution RNA-Bee™ (Cat. No. CS-104B or CS-105B or CS-501B) (AMS Biotechnology, Abingdon, UK) and homogenates were transferred to sterile centrifuge tubes. 60 µl of chloroform (Cat. No. C7559) (Sigma-Aldrich, Poole, UK) was added to the homogenate and samples centrifuged at 4 °C for 15 min at 9,000 g. Supernatants were collected in fresh tubes and a single volume of 70 % ethanol was added. Samples were inverted repeatedly and transferred to spin columns and RNA_{total} extracted using the RNEasy Mini Kit (Cat. No. 74104 or 74106) (Qiagen Ltd., Crawley, UK) according to manufacturer's instructions. Isolated RNA_{total} was suspended in a final volume of 30 µl nuclease-free water (Cat. No. AM9938) (Ambion Inc., Warrington, UK) and stored at -70 °C.

4.3.4 RNA Quantification

The purity and concentration (ng/µl) of RNA_{total} for each sample was determined by calculating the $A_{260}:A_{280}$ and $A_{260}:A_{230}$ ratios using the NanoDrop™ 1000 spectrophotometer (Now replaced by Cat. No. ND-2000, NanoDrop™ 2000 spectrophotometer) (ThermoFisher Scientific, Nottingham, UK), according to manufacturer's instructions. RNA_{total} was then quantified by fluorescence with the Quant-iT™ Ribogreen® RNA Assay Kit (Cat. No. R11490) (Molecular Probes, Invitrogen Ltd., Paisley, UK), according to the manufacturer's protocol.

4.3.5 Reverse-Transcription

50 ng RNA_{total} was converted to cDNA using the WT-Ovation™ Pico RNA Amplification System (Now replaced by Cat. No. 3302, Ovation Pico WTA System V2) (NuGen Technologies, Inc., California, USA) according to the manufacturer's protocol. cDNA was then purified using the DNA Clean and Concentrator 25™ kit (Cat. No. D4033 or D4034) (Zymo Research Corporation, California, USA) according to manufacturer's instructions; and quantified with the NanoDrop™ 1000 spectrophotometer. Samples were stored permanently at -20 °C.

4.3.6 Selection of Angiogenesis-Specific Genes for mRNA Expression

Analysis

The panel of genes thought to play a role in the angiogenic response of the human dental pulp to orthodontic force application was expanded from the panel used in **Chapter 3** in order to link the changes in mRNA expression to known signalling cascades. The extended panel of angiogenesis-specific genes that we selected for investigation is outlined in **Table 34**.

Gene Symbol	Gene Name	Gene Function
ANGPT1	<i>angiopoietin 1</i>	Signal transduction by binding to endothelial-specific tyrosine kinase
FGF2	<i>fibroblast growth factor 2 (basic)</i>	Heparin binding growth factor (mitogenic and angiogenic)
HIF1A	<i>hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)</i>	Activation of signal transduction in homeostatic response to hypoxia
IL8	<i>interleukin 8</i>	Chemotatic and angiogenic factor playing a major role in the inflammatory response
ITGAV	<i>integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)</i>	Cell-surface adhesion receptor and mediator of cell-surface signalling
ITGB3	<i>integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)</i>	Cell-surface adhesion receptor and mediator of cell-surface signalling
KDR	<i>kinase insert domain receptor (a type III receptor tyrosine kinase)</i>	Transmembrane receptor protein tyrosine kinase activity
MMP2	<i>matrix metalloproteinase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)</i>	Metalloendopeptidase activity
NOS2	<i>nitric oxide synthase 2, inducible</i>	Production of nitric oxide; a mediator of the hypoxic response, immune response, endothelial cell proliferation and angiogenesis
PDGFA	<i>platelet-derived growth factor alpha polypeptide</i>	Collagen binding growth factor (mitogenic)
PDGFB	<i>platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)</i>	Collagen binding growth factor (mitogenic)
ROBO4	<i>roundabout homolog 4, magic roundabout (Drosophila)</i>	Regulation of cell migration in angiogenesis and vascular patterning
SELE	<i>selectin E</i>	Cell-surface glycoprotein playing a mediatory role in immunoadhesion
TEK	<i>TEK tyrosine kinase, endothelial</i>	Transmembrane receptor protein tyrosine kinase activity
TGFB1	<i>transforming growth factor, beta 1</i>	Multifunctional mitogenic growth factor
TGFBR1	<i>transforming growth factor, beta receptor 1</i>	Transmembrane receptor protein serine/threonine kinase activity
VEGFA	<i>vascular endothelial growth factor A</i>	Endothelial-specific heparin and heparin sulphate binding growth factor (mitogenic and angiogenic)

Table 34 Expanded list of angiogenesis-specific genes for mRNA expression analysis

4.3.7 RT-qPCR Analysis of mRNA Expression

RT-qPCR was performed on 20 µl reactions set up manually in 0.1 ml strip tubes (Cat. No. 981103) (Qiagen Ltd., Crawley, UK): 5 µl cDNA (2 ng/µl), 1 µl forward and reverse primer mix (6 µM) (designed and optimised by PrimerDesign Ltd., Southampton, UK; see **Table 35 – Table 51**), 10 µl Precision Mastermix with SYBR Green (Now replaced by Cat. No. PrecisionPlus-SY, PrecisionPlus™ Mastermix) (PrimerDesign Ltd., Southampton, UK) and 4 µl RNase/DNase-free water (delivered in conjunction with the primers, no individual Cat. No.) (PrimerDesign Ltd., Southampton, UK) with the Corbett Rotor-Gene™ 6000 (No Cat. No. available but item is still available from Qiagen) (Corbett Life Science, Qiagen Ltd., Crawley, UK). Amplifications were performed with an initial template denaturation step at 95 °C for 10 min followed by 50 cycles of denaturation at 95 °C for 15 sec; combined primer annealing/extension at 60 °C for 60 sec; and data acquisition.

A dissociation protocol was performed from 72 °C to 95 °C at 1 °C increments in order to determine the presence of primer-dimer formation or other spurious binding products. In order to determine the size of the amplification products, a single assay was performed for each angiogenesis-specific GOI without a dissociation protocol. 5 µl RT-qPCR product and 0.83 µl 6 × blue/orange loading dye (Cat. No. G1881) (Promega UK Ltd., UK) were loaded onto a 2 % agarose gel made with 0.5 × TBE buffer and 2 µl GelRed™ Nucleic Acid Gel Stain (Cat. No. 41003) (Biotium Inc., Cambridge BioScience Ltd., Cambridge, UK). Samples were run in 0.5 × TBE buffer for 15 min at 100 V alongside a 100 bp ladder and gels were photographed using a UV transilluminator linked to a printer to confirm primer specificity.

Primers for RPL13A and UBC, established as the most stable expressed reference genes in our experimental model (see **Chapter 3**), were designed, optimised by and

guaranteed a reaction efficiency of 100 % by Primer Design Ltd., Southampton, UK; primer sequences were not given.

Primers for the angiogenesis-specific GOIs were designed, optimised by and guaranteed a reaction efficiency of 100 % by Primer Design Ltd., Southampton, UK The details for these primers are provided in **Table 35 - Table 51**.

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
ANGPT1	NM_001146.4	107	1.00
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
544 - 563	650 - 628	Forward primer	ACATAGGGTGCAGCAATCAG
Location of Primer by Exon/Intron		Reverse primer	ATCGTGTCTGGAAGAATGAAAG
Spans part of exon 1			
Secondary Structure of Amplicon		Splice Variants Targeted	
None		ANGPT1 - 001 and ANGPT1 - 003	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
None listed in NCBI database		http://www.ncbi.nlm.nih.gov/homologene/37447	

Table 35 PrimerDesign Ltd. UK primer sequences of the angiogenesis-specific gene ANGPT1 for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
FGF2	NM_002006.4	137	1.00
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
5049 - 5073	5185 - 5160	Forward primer	ATAGTGTGAGAGAATTAGGCTGTAG
Location of Primer by Exon/Intron		Reverse primer	AATCTTGGTTTCAGGTACTATTGAC
Spans part of exon 3			
Secondary Structure of Amplicon		Splice Variants Targeted	
None		FGF2 - 001 and FGF2 - 201	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
None listed in NCBI database		http://www.ncbi.nlm.nih.gov/homologene/1521	

Table 36 PrimerDesign Ltd. UK primer sequences of the angiogenesis-specific gene FGF2 for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
HIF1A	NM_001530.3	132	1.00
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
2363 - 2385	2494 - 2473		
Location of Primer by Exon/Intron		Forward primer	TGCCACATCATCACCATATAGAG
Spans part of exon 12 and 13		Reverse primer	TGACTCAAAGCGACAGATAACA
Secondary Structure of Amplicon		Splice Variants Targeted	
None		HIF1A - 001, HIF1A - 002, HIF1A - 003, HIF1A - 004, HIF1A - 011 and HIF1A - 201	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
http://www.ncbi.nlm.nih.gov/gene/?Term=related_functional_gene_3091[group]		http://www.ncbi.nlm.nih.gov/homologene/1171	

Table 37 PrimerDesign Ltd. UK primer sequences of the angiogenesis-specific gene HIF1A for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
IL8	NM_000584.2	128	1.00
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
425 - 446	552 - 531		
Location of Primer by Exon/Intron		Forward primer	AGTTTTGAAGAGGGCTGAGAA
Spans part of exon 3 and 4		Reverse primer	ACAACAGACCCACACAATACAT
Secondary Structure of Amplicon		Splice Variants Targeted	
None		CXCL8 - 001	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
None listed in NCBI database		http://www.ncbi.nlm.nih.gov/homologene/47937	

Table 38 PrimerDesign Ltd. UK primer sequences of the angiogenesis-specific gene IL8 for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
ITGAV	NM_002210.4	121	1.00
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
6195 - 6219	6315 - 6294	Forward primer	AAACAGAATTTGTAAGTTGGCAGAT
Location of Primer by Exon/Intron		Reverse primer	GGTGACATTGAGATGGGTAGTG
Spans part of exon 30			
Secondary Structure of Amplicon		Splice Variants Targeted	
None		ITGAV - 001 and ITGAV - 003	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
None listed in NCBI database		http://www.ncbi.nlm.nih.gov/homologene/20510	

Table 39 PrimerDesign Ltd. UK primer sequences of the angiogenesis-specific gene ITGAV for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
ITGB3	NM_000212.2	120	1.00
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
3218 - 3241	3337 - 3316	Forward primer	ACCATCTCTTACCTCCTAATTCC
Location of Primer by Exon/Intron		Reverse primer	CTGGCTCTACAATAGCACTCTC
Spans part of exon 15			
Secondary Structure of Amplicon		Splice Variants Targeted	
None		ITGB3 - 001	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
None listed in NCBI database		http://www.ncbi.nlm.nih.gov/homologene/55444	

Table 40 PrimerDesign Ltd. UK primer sequences of the angiogenesis-specific gene ITGB3 for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
KDR	NM_002253.2	77	1.00
Location of Amplicon		Primer Sequence	
Plus Strand 4173 - 4191	Minus Strand 4249 - 4230	Sequence (5'→3')	
Location of Primer by Exon/Intron Spans part of exon 29 and 30		Forward primer	AGGGAGTCTGTGGCATCTG
		Reverse primer	GTGTCTGTGCATCGGAGTG
Secondary Structure of Amplicon None		Splice Variants Targeted KDR - 001	
Pseudogenes or retropseudogenes listed at: None listed in NCBI database		Homologs listed at: http://www.ncbi.nlm.nih.gov/homologene/55639	

Table 41 PrimerDesign Ltd. UK primer sequences of the angiogenesis-specific gene KDR for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
MMP2	NM_004530.5	90	1.00
Location of Amplicon		Primer Sequence	
Plus Strand 685 - 706	Minus Strand 774 - 756	Sequence (5'→3')	
Location of Primer by Exon/Intron Spans part of exon 2 and 3		Forward primer	CATACAGGATCATTGGCTACAC
		Reverse primer	TCACATCGTCCAGACTTG
Secondary Structure of Amplicon None		Splice Variants Targeted MMP2 - 001, MMP2 - 002, MMP2 - 003, MMP2 - 004, MMP2 - 007 and MMP2 - 008	
Pseudogenes or retropseudogenes listed at: None listed in NCBI database		Homologs listed at: http://www.ncbi.nlm.nih.gov/homologene/3329	

Table 42 PrimerDesign Ltd. UK primer sequences of the angiogenesis-specific gene MMP2 for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
NOS2	NM_000625.4	91	1.00
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
726 - 749	816 - 797	Forward primer	CAAAGAGGCAAAATAGAGGAACA
Location of Primer by Exon/Intron		Reverse primer	ATCTCCCGTCAGTTGGTAGG
Spans part of exon 5 and 6			
Secondary Structure of Amplicon		Splice Variants Targeted	
None		NOS2 - 001 and NOS2 - 201	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
http://www.ncbi.nlm.nih.gov/gene/?Term=related_functional_gene_4843[group]		http://www.ncbi.nlm.nih.gov/homologene/55473	

Table 43 PrimerDesign Ltd. UK primer sequences of the angiogenesis-specific gene NOS2 for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
PDGFA	NM_002607.5	80	1.00
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
1481 - 1499	1560 - 1540	Forward primer	GCAGCCAACCAGATGTGAG
Location of Primer by Exon/Intron		Reverse primer	TCAGGAATGTAACACGCCATG
Spans part of exon 6 and 7			
Secondary Structure of Amplicon		Splice Variants Targeted	
None		PDGFA - 001, PDGFA - 002 and PDGFA - 005	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
None listed in NCBI database		http://www.ncbi.nlm.nih.gov/homologene/32055	

Table 44 PrimerDesign Ltd. UK primer sequences of the angiogenesis-specific gene PDGFA for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
PDGFB	NM_002608.3	89	1.00
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
3300 - 3318	3388 - 3362	Forward primer	ACCTCCCTTCCCACCTACT
Location of Primer by Exon/Intron		Reverse primer	CCAAAAATATAATCACCGACTTTACAA
Spans part of exon 7			
Secondary Structure of Amplicon		Splice Variants Targeted	
None		PDGFB - 001	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
None listed in NCBI database		http://www.ncbi.nlm.nih.gov/homologene/74303	

Table 45 PrimerDesign Ltd. UK primer sequences of the angiogenesis-specific gene PDGFB for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
ROBO4	NM_019055.5	91	1.00
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
3138 - 3160	3228 - 3210	Forward primer	GGTGCTTCTCCTGTAGATTACTC
Location of Primer by Exon/Intron		Reverse primer	AGGTCTTGTGGGTGGACAG
Spans part of exon 17 and 18			
Secondary Structure of Amplicon		Splice Variants Targeted	
None		ROBO4 - 001, ROBO4 - 003 and ROBO4 - 007	
Pseudogenes or retropseudogenes listed at:		Homologs listed at:	
None listed in NCBI database		http://www.ncbi.nlm.nih.gov/homologene/10397	

Table 46 PrimerDesign Ltd. UK primer sequences of the angiogenesis-specific gene ROBO4 for RT-qPCR

Gene Symbol SELE	Accession Number NM_000450.2	Amplicon Length 123	Reaction Efficiency 1.00
Location of Amplicon		Primer Sequence	
Plus Strand 2083 - 2104	Minus Strand 2205 - 2185	Sequence (5'→3')	
Location of Primer by Exon/Intron Spans part of exon 14		Forward primer	TTCTTGCCTACTATGCCAGATG
		Reverse primer	AGGAAAGGGAACACTGAGTCT
Secondary Structure of Amplicon None		Splice Variants Targeted SELE - 001, SELE - 002, SELE - 003 and SELE - 005	
Pseudogenes or retropseudogenes listed at: None listed in NCBI database		Homologs listed at: http://www.ncbi.nlm.nih.gov/homologene/389	

Table 47 PrimerDesign Ltd. UK primer sequences of the angiogenesis-specific gene SELE for RT-qPCR

Gene Symbol TEK	Accession Number NM_000459.4	Amplicon Length 147	Reaction Efficiency 1.00
Location of Amplicon		Primer Sequence	
Plus Strand 302 - 322	Minus Strand 448 - 428	Sequence (5'→3')	
Location of Primer by Exon/Intron Spans part of exon 1		Forward primer	GCTTCTGTGCTGTTCTTCTT
		Reverse primer	TTCCCCAAATCTCTCCATCCA
Secondary Structure of Amplicon None		Splice Variants Targeted TEK - 002, TEK - 003, TEK - 005 and TEK - 201	
Pseudogenes or retropseudogenes listed at: None listed in NCBI database		Homologs listed at: http://www.ncbi.nlm.nih.gov/homologene/397	

Table 48 PrimerDesign Ltd. UK primer sequences of the angiogenesis-specific gene TEK for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
TGFB1	NM_000660.5	83	1.00
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
2258 - 2276	2340 - 2323		
Location of Primer by Exon/Intron Spans part of exon 7		Forward primer	CACTCCCACTCCCTCTCTC
		Reverse primer	GTCCCCTGTGCCTTGATG
Secondary Structure of Amplicon None		Splice Variants Targeted TGFB1 - 001 and TGFB1 - 004	
Pseudogenes or retropseudogenes listed at: None listed in NCBI database		Homologs listed at: http://www.ncbi.nlm.nih.gov/homologene/540	

Table 49 PrimerDesign Ltd. UK primer sequences of the angiogenesis-specific gene TGFB1 for RT-qPCR

Gene Symbol	Accession Number	Amplicon Length	Reaction Efficiency
TGFBR1	NM_004612.3	125	1.00
Location of Amplicon		Primer Sequence	
Plus Strand	Minus Strand	Sequence (5'→3')	
4450 - 4468	4574 - 4552		
Location of Primer by Exon/Intron Spans part of exon 9		Forward primer	TGACTGAAGGCTGCTCTGG
		Reverse primer	CATCTGCTCAATCTCCAACTTG
Secondary Structure of Amplicon None		Splice Variants Targeted TGFBR1 - 001, TGFBR1 - 003 and TGFBR1 - 004	
Pseudogenes or retropseudogenes listed at: http://www.ncbi.nlm.nih.gov/gene/?Term=related_functional_gene_7046[group]		Homologs listed at: http://www.ncbi.nlm.nih.gov/homologene/3177	

Table 50 PrimerDesign Ltd. UK primer sequences of the angiogenesis-specific gene TGFBR1 for RT-qPCR

Gene Symbol VEGFA	Accession Number NM_001025366.2	Amplicon Length 93	Reaction Efficiency 1.00
Location of Amplicon		Primer Sequence	
Plus Strand 1790 - 1812	Minus Strand 1882 - 1860	Sequence (5'→3')	
Location of Primer by Exon/Intron Spans part of exon 8		Forward primer	CCAGGAAAGACTGATACAGAACG
		Reverse primer	GGTTTCTGGATTAAGGACTGTTTC
Secondary Structure of Amplicon None		Splice Variants Targeted VEGFA - 001, VEGFA - 002, VEGFA - 003, VEGFA - 007, VEGFA - 008, VEGFA - 009, VEGFA - 018, VEGFA - 019, VEGFA - 020, VEGFA - 201, VEGFA - 202, VEGFA - 203 and VEGFA - 204	
Pseudogenes or retropseudogenes listed at: None listed in NCBI database		Homologs listed at: http://www.ncbi.nlm.nih.gov/homologene/2534	

Table 51 PrimerDesign Ltd. UK primer sequences of the angiogenesis-specific gene VEGFA for RT-qPCR

4.3.8 Statistical Analysis of RT-qPCR Data

Raw RT-qPCR data was analysed with Corbett Rotor-Gene™ 6000 Application Software, version 1.7 (Build 87) and expressed as quantification cycle (C_q) values. All C_q values of unknowns fell within the linear quantifiable range of the reaction. Relative mRNA expression of the GOIs was compared between ‘control’ and contralateral ‘treated’ pulps using the REST-2009© software package (developed by Prof. M. Pfaffl, Technical University Munich, Germany and Qiagen Ltd., Crawley, UK; <http://www.gene-quantification.com/rest-2009.html>). Statistical significance was given at $p \leq 0.05$.

Data was normalised against the geometric mean expression of the reference genes RPL13A and UBC, previously established to be the most stably expressed pair of reference genes in our experimental system (see **Chapter 3**).

Reaction efficiencies were assumed to be 100 %, as guaranteed by PrimerDesign Ltd., Southampton, UK

In order to eliminate the effect of individual variability on average expression values, C_q values were analysed by the statistical tests outlined in ‘Standardization of real-time PCR gene expression data from independent biological replicates’ by Willems *et al* (2008); namely log transformation, mean centring and autoscaling. Methodology was clarified by Dr Nora Donaldson, Statistical Advisor, Dental Institute, King’s College London.

In order to identify patterns on an inter- and intra-patient basis and to determine whether patient age, ethnicity, gender or root morphology was linked to gene expression profiles, data was sent to Dr Nora Donaldson, Statistical Advisor, Dental Institute, King’s College London for analysis.

Statistical significance was given at $p \leq 0.05$.

4.3.9 Pulp Tissue Homogenisation for Protein Analysis

Individual snap-frozen pulp samples were homogenised in 2 ml sterile centrifuge tubes containing 250 μ l lysis buffer (Cat. No. EL-lysis) (RayBiotech Inc., Georgia, USA) supplemented with 25 μ l protease inhibitor cocktail (Cat. No. 539131) (Calbiochem®, Merck4Biosciences, Darmstadt, Germany) with an Ultra-Turrax® homogeniser (Cat. No. 3370100) (Rose Scientific Ltd., Alberta, Canada). Tissue lysates were centrifuged at 4 °C for 10 min at 5,000 g to separate tissue debris from supernatants; debris pellets were discarded and tissue lysate supernatants were stored on ice.

The total protein concentration was determined with the Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Cat. No. 23250) (ThermoFisher Scientific, Essex, UK), according to manufacturer’s instructions.

Samples were diluted to a concentration of 2 µg/µl with lysis buffer supplemented with protease inhibitor cocktail (1:10 dilution) as per instruction from RayBiotech Inc., Georgia, USA before snap-freezing 1000 µl aliquots in liquid nitrogen and transferring to -70 °C for permanent storage.

4.3.10 Quantification of Proteins Associated with Angiogenesis

Frozen human dental pulp lysates (1000 µl at a concentration of 2 µg/µl) were delivered to RayBiotech Inc., Georgia, USA for Quantibody® Array Testing. 12.5 µl of the human dental pulp lysate was added to each well of a custom Quantibody® multiplex sandwich-based Enzyme-Linked ImmunoSorbent Assay (ELISA) array in addition to 12.5 µl running buffer, making a total concentration of 1 µg/µl. Reactions were performed in technical quadruplicate. The custom Quantibody® ELISA array targeted the following angiogenesis associated proteins: ANGPT1; FGF2; KDR; MMP2; PDGFA; PDGFB; SELE; TEK; TGFB1; VEGFA. Analyte concentrations were determined and delivered in a service report.

4.4 Results

4.4.1 Relative mRNA Expression Analysis of RT-qPCR Data

RT-qPCR was performed in technical duplicate using primers for the angiogenesis-specific genes: ANGPT1, FGF2, HIF1A, IL8, ITGAV, ITGB3, KDR, MMP2, NOS2, PDGFA, PDGFB, ROBO4, SELE, TEK, TGFB1, TGFBR1 and VEGFA. RT-qPCR was performed on cDNA from isolated pulp samples that had been treated with an orthodontic force for 1 day (n = 10), 3 days (n = 10) or 14 days (n = 10) prior to tooth extraction; and on the untreated, contralaterally paired intra-patient controls (n = 10 per treatment time-point). C_q values were input into REST-2009© software and expression ratios were calculated, normalising against the geometric mean expression of reference genes RPL13A and UBC.

There was shown to be no significant change in the mRNA expression of any of the angiogenesis-specific GOIs in human dental pulps treated with an orthodontic force for a period of 1 day prior to extraction (**Figure 14**).

Following 3 days of orthodontic force application, there was a significant down-regulation, by a mean factor of 0.531, of the mRNA expression of TEK. There was no significant difference in the mRNA expression of any of the other GOIs in treated samples relative to the untreated controls (**Figure 15**).

There was no significant up- or down-regulation of mRNA expression for any of the GOIs following 14 days of orthodontic force application prior to extraction (**Figure 16**).

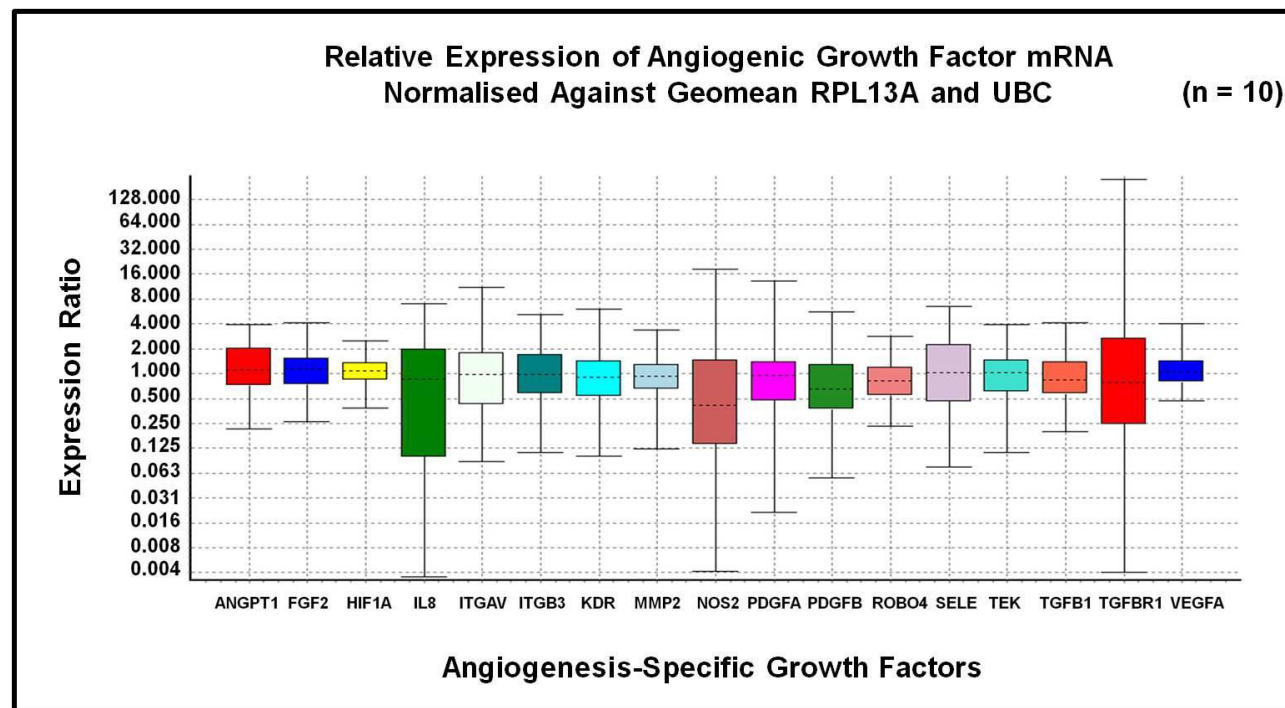


Figure 14 Differential expression analysis of angiogenic growth factor mRNA in one day treated samples relative to contralateral control. Normalising against the geometric mean of RPL13A and UBC

Plot shows expression ratios calculated by REST-2009© software. Box-Whisker plots illustrate the range of observations (whiskers), 25th and 75th percentiles (boxes) and sample medians (dashed line). Data was normalised against the geometric mean of RPL13A and UBC, which has been identified as a suitable normalisation factor under our experimental conditions. There was no significant difference ($p > 0.05$) in mRNA expression of any of the GOIs in the one day treated samples (n = 10) relative to their paired contralateral controls (n = 10).

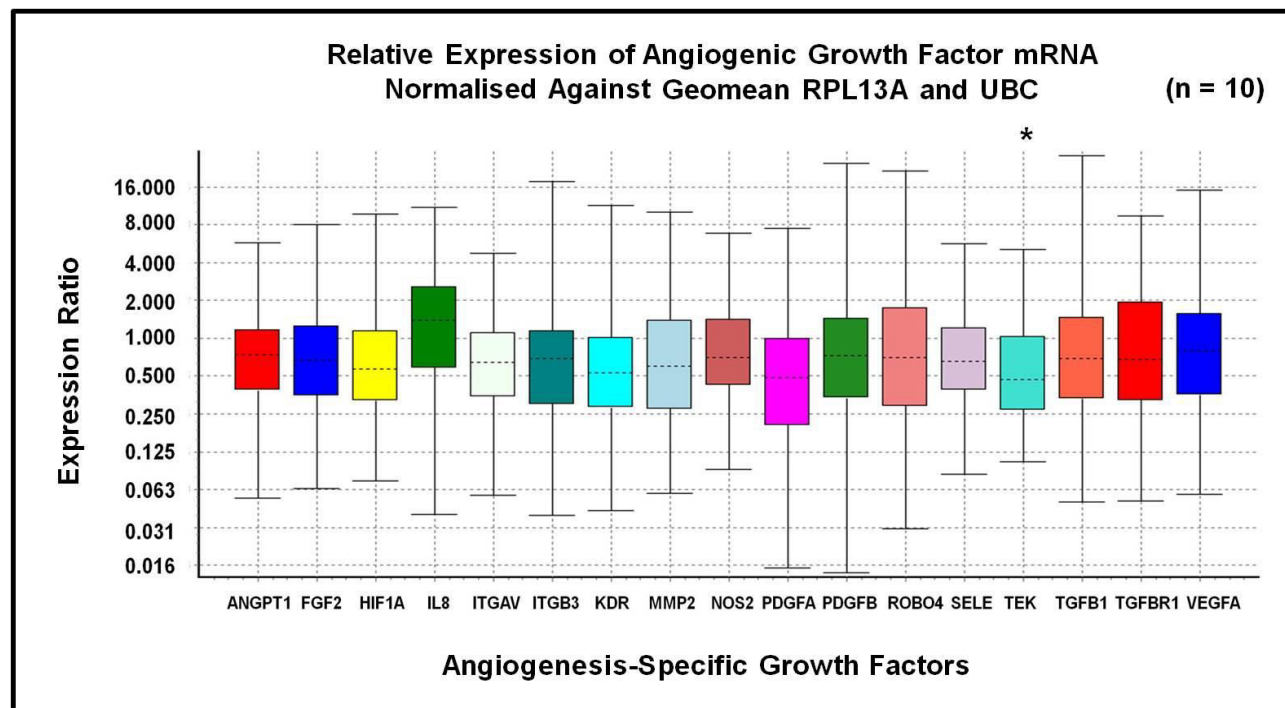


Figure 15 Differential expression analysis of angiogenic growth factor mRNA expression in three day treated samples relative to contralateral controls. Normalising against the geometric mean of RPL13A and UBC

Plot shows expression ratios calculated by REST-2009© software. Box-Whisker plots illustrate the range of observations (whiskers), 25th and 75th percentiles (boxes) and sample medians (dashed line). Data was normalised against the geometric mean of RPL13A and UBC, which has been identified as a suitable normalisation factor under our experimental conditions. There was a significant down-regulation of TEK mRNA expression in the three day treated samples (n = 10) relative to their paired contralateral controls (n = 10).

* indicates statistical significance ($p \leq 0.05$).

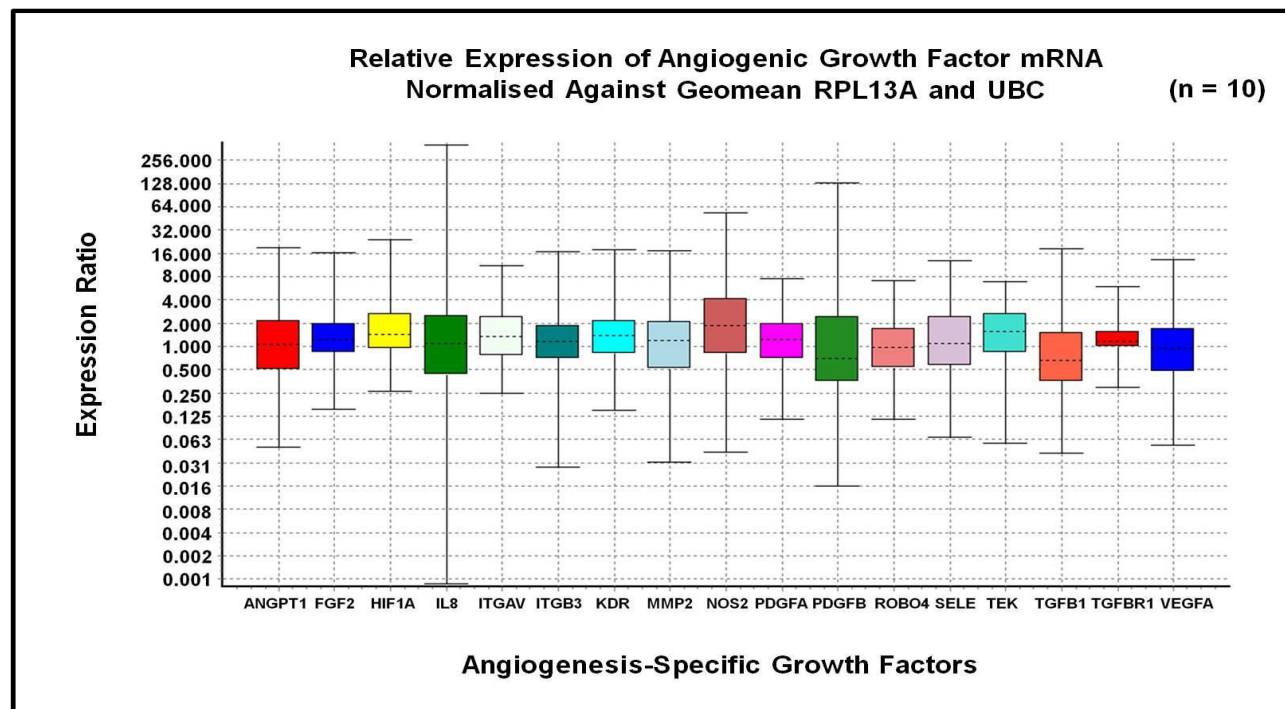


Figure 16 Differential expression analysis of angiogenic growth factor mRNA expression in fourteen day treated samples relative to contralateral controls. Normalising against the geometric mean of RPL13A and UBC

Plot shows expression ratios calculated by REST-2009© software. Box-Whisker plots illustrate the range of observations (whiskers), 25th and 75th percentiles (boxes) and sample medians (dashed line). Data was normalised against the geometric mean of RPL13A and UBC, which has been identified as a suitable normalisation factor under our experimental conditions. There was no significant difference ($p > 0.05$) in mRNA expression of any of the GOIs in the fourteen day treated samples ($n = 10$) relative to their paired contralateral controls ($n = 10$).

4.4.2 Assessment of Biological Variability in RT-qPCR Data

In order to assess the level of variability in gene expression amongst the untreated ‘control’ pulps and therefore the inter-patient biological variability, raw C_q values were log-transformed and the mean, standard error of the mean, standard deviation and coefficient of variation were calculated for each angiogenesis-specific GOI. Results are shown in **Table 52 – Table 57**.

Untreated ‘control’ pulp samples should demonstrate similar gene expression patterns as all samples were of approximately the same size, had a similar morphology and were extracted from patients within a small age range. All samples were isolated from teeth with sound dental history and were not exposed to orthodontic force prior to extraction. All genes showed a coefficient of variation (%) in the range of 41.14 % – 124.15 %.

ANGPT1					
2 Weeks		3 Days		1 Day	
Control 1	2.657E-05	Control 1	1.905E-05	Control 1	1.194E-05
Control 2	2.077E-05	Control 2	2.234E-05	Control 2	1.227E-05
Control 3	6.900E-06	Control 3	4.301E-05	Control 3	2.760E-05
Control 4	2.056E-05	Control 4	2.049E-05	Control 4	8.262E-06
Control 5	4.862E-06	Control 5	1.125E-05	Control 5	1.366E-05
Control 6	2.173E-05	Control 6	1.464E-05	Control 6	2.034E-05
Control 7	1.227E-05	Control 7	3.351E-05	Control 7	1.244E-05
Control 8	6.483E-06	Control 8	1.409E-05	Control 8	5.016E-06
Control 9	4.262E-06	Control 9	4.055E-05	Control 9	7.094E-06
Control 10	9.873E-07	Control 10	3.271E-05	Control 10	4.088E-06
Mean \pm SEM	1.666E-05 \pm 1.997E-06	SD	1.094E-05	% CV	65.660
FGF2					
2 Weeks		3 Days		1 Day	
Control 1	3.990E-06	Control 1	2.491E-06	Control 1	2.707E-06
Control 2	4.490E-06	Control 2	3.241E-06	Control 2	4.074E-06
Control 3	2.191E-06	Control 3	6.805E-06	Control 3	2.972E-06
Control 4	1.862E-06	Control 4	3.710E-06	Control 4	1.368E-06
Control 5	3.698E-06	Control 5	5.546E-06	Control 5	2.044E-06
Control 6	2.348E-06	Control 6	4.947E-06	Control 6	1.749E-06
Control 7	1.695E-06	Control 7	4.862E-06	Control 7	9.148E-07
Control 8	1.241E-06	Control 8	4.032E-06	Control 8	1.792E-06
Control 9	1.073E-06	Control 9	7.193E-06	Control 9	1.678E-06
Control 10	1.604E-06	Control 10	6.154E-06	Control 10	1.095E-06
Mean \pm SEM	3.119E-06 \pm 3.239E-07	SD	1.774E-06	% CV	56.889
HIF1A					
2 Weeks		3 Days		1 Day	
Control 1	3.469E-05	Control 1	2.250E-05	Control 1	3.542E-05
Control 2	3.530E-05	Control 2	3.567E-05	Control 2	3.796E-05
Control 3	1.394E-05	Control 3	4.923E-05	Control 3	3.457E-05
Control 4	3.116E-05	Control 4	1.802E-05	Control 4	1.057E-05
Control 5	2.666E-05	Control 5	1.993E-05	Control 5	1.310E-05
Control 6	1.222E-05	Control 6	2.847E-05	Control 6	9.168E-06
Control 7	1.173E-05	Control 7	2.611E-05	Control 7	7.193E-06
Control 8	1.444E-05	Control 8	2.847E-05	Control 8	1.189E-05
Control 9	8.291E-06	Control 9	3.305E-05	Control 9	7.817E-06
Control 10	1.333E-05	Control 10	2.798E-05	Control 10	7.447E-06
Mean \pm SEM	2.221E-05 \pm 2.128E-06	SD	1.165E-05	% CV	52.466

Table 52 Variability of ANGPT1, FGF2 and HIF1A mRNA Expression in Control Samples

C_q values were log transformed in order to represent the mean, the standard error of the mean, the standard deviation and the coefficient of variation for all control samples used in the orthodontic experiments.

IL8					
2 Weeks		3 Days		1 Day	
Control 1	1.185E-08	Control 1	3.205E-08	Control 1	3.606E-08
Control 2	2.625E-09	Control 2	1.112E-07	Control 2	4.660E-08
Control 3	1.255E-09	Control 3	2.040E-07	Control 3	3.798E-08
Control 4	9.675E-10	Control 4	7.237E-08	Control 4	1.244E-08
Control 5	6.495E-10	Control 5	3.569E-08	Control 5	2.064E-08
Control 6	3.192E-10	Control 6	5.153E-08	Control 6	1.415E-08
Control 7	1.155E-09	Control 7	5.523E-08	Control 7	5.054E-09
Control 8	2.280E-10	Control 8	3.365E-08	Control 8	3.720E-08
Control 9	3.764E-09	Control 9	5.409E-08	Control 9	7.024E-09
Control 10	7.730E-08	Control 10	9.034E-08	Control 10	1.405E-08
Mean \pm SEM	3.572E-08 \pm 7.927E-09	SD	4.342E-08	% CV	121.566
ITGAV					
2 Weeks		3 Days		1 Day	
Control 1	7.208E-05	Control 1	9.251E-05	Control 1	1.135E-04
Control 2	1.163E-04	Control 2	1.447E-04	Control 2	2.294E-04
Control 3	1.442E-04	Control 3	1.633E-04	Control 3	2.239E-04
Control 4	1.482E-04	Control 4	1.350E-04	Control 4	4.459E-06
Control 5	1.327E-04	Control 5	1.317E-04	Control 5	8.495E-06
Control 6	2.053E-04	Control 6	1.656E-04	Control 6	1.064E-05
Control 7	1.238E-04	Control 7	7.888E-05	Control 7	1.181E-05
Control 8	1.089E-04	Control 8	1.304E-04	Control 8	1.840E-05
Control 9	9.644E-05	Control 9	1.402E-04	Control 9	8.703E-06
Control 10	8.194E-05	Control 10	1.605E-04	Control 10	1.140E-05
Mean \pm SEM	1.071E-04 \pm 1.197E-05	SD	6.554E-05	% CV	61.188
ITGB3					
2 Weeks		3 Days		1 Day	
Control 1	2.168E-06	Control 1	1.186E-06	Control 1	2.153E-06
Control 2	2.206E-06	Control 2	1.792E-06	Control 2	3.462E-06
Control 3	1.823E-06	Control 3	3.264E-06	Control 3	1.981E-06
Control 4	2.146E-06	Control 4	2.773E-06	Control 4	1.312E-06
Control 5	1.773E-06	Control 5	2.952E-06	Control 5	1.941E-06
Control 6	2.365E-06	Control 6	2.851E-06	Control 6	7.227E-07
Control 7	1.146E-06	Control 7	3.131E-06	Control 7	7.253E-07
Control 8	5.458E-07	Control 8	6.028E-06	Control 8	2.633E-06
Control 9	2.044E-06	Control 9	2.482E-06	Control 9	1.786E-06
Control 10	6.028E-06	Control 10	5.034E-06	Control 10	8.625E-07
Mean \pm SEM	2.377E-06 \pm 2.496E-07	SD	1.367E-06	% CV	57.512

Table 53 Variability of IL8, ITGAV and ITGB3 mRNA Expression in Control Samples

C_q values were log transformed in order to represent the mean, the standard error of the mean, the standard deviation and the coefficient of variation for all control samples used in the orthodontic experiments.

KDR					
2 Weeks		3 Days		1 Day	
Control 1	7.094E-06	Control 1	5.086E-06	Control 1	9.557E-06
Control 2	7.871E-06	Control 2	9.200E-06	Control 2	8.092E-06
Control 3	3.510E-06	Control 3	8.979E-06	Control 3	5.624E-06
Control 4	3.828E-06	Control 4	5.508E-06	Control 4	3.584E-06
Control 5	6.007E-06	Control 5	5.034E-06	Control 5	6.596E-06
Control 6	6.197E-06	Control 6	9.963E-06	Control 6	4.616E-06
Control 7	5.284E-06	Control 7	9.360E-06	Control 7	6.550E-06
Control 8	2.292E-06	Control 8	1.129E-05	Control 8	6.900E-06
Control 9	8.584E-06	Control 9	5.782E-06	Control 9	4.521E-06
Control 10	4.103E-06	Control 10	1.449E-05	Control 10	2.962E-06
Mean ± SEM	6.615E-06 ± 4.969E-07	SD	2.721E-06	% CV	41.139
MMP2					
2 Weeks		3 Days		1 Day	
Control 1	1.456E-06	Control 1	2.597E-06	Control 1	3.241E-06
Control 2	1.549E-06	Control 2	1.725E-06	Control 2	5.034E-06
Control 3	2.253E-06	Control 3	2.080E-06	Control 3	6.596E-06
Control 4	4.018E-06	Control 4	1.401E-06	Control 4	1.272E-06
Control 5	2.822E-06	Control 5	1.150E-06	Control 5	1.927E-06
Control 6	1.649E-06	Control 6	2.754E-06	Control 6	8.216E-07
Control 7	4.194E-07	Control 7	3.474E-06	Control 7	4.418E-07
Control 8	8.302E-07	Control 8	3.109E-06	Control 8	1.182E-06
Control 9	1.660E-06	Control 9	2.465E-06	Control 9	1.088E-06
Control 10	2.633E-06	Control 10	2.356E-06	Control 10	5.182E-07
Mean ± SEM	2.151E-06 ± 2.512E-07	SD	1.376E-06	% CV	63.964
NOS2					
2 Weeks		3 Days		1 Day	
Control 1	8.069E-09	Control 1	3.865E-08	Control 1	5.268E-09
Control 2	1.736E-08	Control 2	5.981E-08	Control 2	1.187E-09
Control 3	2.577E-08	Control 3	4.825E-08	Control 3	6.353E-09
Control 4	9.015E-09	Control 4	4.470E-08	Control 4	9.608E-10
Control 5	2.355E-08	Control 5	4.995E-08	Control 5	2.509E-09
Control 6	6.322E-08	Control 6	2.282E-08	Control 6	1.410E-08
Control 7	1.106E-08	Control 7	5.542E-08	Control 7	1.057E-08
Control 8	1.444E-08	Control 8	4.363E-08	Control 8	7.985E-09
Control 9	4.564E-08	Control 9	3.239E-08	Control 9	1.989E-09
Control 10	4.303E-08	Control 10	4.259E-08	Control 10	1.277E-09
Mean ± SEM	2.505E-08 ± 3.684E-09	SD	2.018E-08	% CV	80.547

Table 54 Variability of KDR, MMP2 and NOS2 mRNA Expression in Control Samples

C_q values were log transformed in order to represent the mean, the standard error of the mean, the standard deviation and the coefficient of variation for all control samples used in the orthodontic experiments.

PDGFA					
2 Weeks		3 Days		1 Day	
Control 1	3.503E-07	Control 1	3.360E-07	Control 1	1.998E-07
Control 2	3.515E-07	Control 2	4.166E-07	Control 2	7.544E-08
Control 3	4.496E-07	Control 3	7.153E-07	Control 3	1.463E-07
Control 4	7.640E-07	Control 4	4.480E-07	Control 4	1.551E-07
Control 5	6.314E-07	Control 5	3.201E-07	Control 5	3.360E-07
Control 6	2.573E-07	Control 6	6.446E-07	Control 6	2.777E-07
Control 7	2.248E-07	Control 7	5.911E-07	Control 7	2.240E-07
Control 8	1.970E-07	Control 8	4.954E-07	Control 8	2.263E-07
Control 9	2.511E-07	Control 9	1.029E-06	Control 9	2.171E-07
Control 10	1.893E-08	Control 10	7.405E-07	Control 10	2.026E-07
Mean ± SEM	3.764E-07 ± 4.273E-08	SD	2.340E-07	% CV	62.172
PDGFB					
2 Weeks		3 Days		1 Day	
Control 1	1.428E-07	Control 1	1.573E-07	Control 1	1.144E-07
Control 2	1.323E-07	Control 2	3.833E-07	Control 2	1.546E-07
Control 3	5.047E-08	Control 3	5.218E-07	Control 3	3.552E-07
Control 4	1.327E-07	Control 4	1.300E-07	Control 4	8.668E-08
Control 5	1.617E-07	Control 5	1.300E-07	Control 5	1.432E-07
Control 6	1.221E-07	Control 6	4.010E-07	Control 6	7.571E-08
Control 7	6.410E-08	Control 7	3.049E-07	Control 7	3.049E-07
Control 8	4.676E-08	Control 8	1.950E-07	Control 8	1.519E-07
Control 9	3.314E-07	Control 9	5.200E-07	Control 9	5.447E-08
Control 10	3.767E-07	Control 10	1.725E-06	Control 10	4.742E-08
Mean ± SEM	2.506E-07 ± 5.680E-08	SD	3.111E-07	% CV	124.148
ROBO4					
2 Weeks		3 Days		1 Day	
Control 1	2.153E-06	Control 1	9.405E-07	Control 1	2.552E-06
Control 2	1.040E-06	Control 2	1.649E-06	Control 2	3.438E-06
Control 3	1.416E-06	Control 3	1.615E-06	Control 3	4.337E-06
Control 4	1.743E-06	Control 4	9.537E-07	Control 4	5.973E-07
Control 5	3.219E-06	Control 5	8.103E-07	Control 5	1.476E-06
Control 6	1.040E-06	Control 6	2.276E-06	Control 6	8.595E-07
Control 7	2.161E-06	Control 7	2.356E-06	Control 7	1.396E-06
Control 8	5.402E-07	Control 8	2.398E-06	Control 8	1.220E-06
Control 9	4.616E-06	Control 9	4.046E-06	Control 9	1.203E-06
Control 10	9.703E-07	Control 10	4.552E-06	Control 10	3.431E-07
Mean ± SEM	1.931E-06 ± 2.258E-07	SD	1.237E-06	% CV	64.071

Table 55 Variability of PDGFA, PDGFB and ROBO4 mRNA Expression in Control Samples

C_q values were log transformed in order to represent the mean, the standard error of the mean, the standard deviation and the coefficient of variation for all control samples used in the orthodontic experiments.

SELE					
2 Weeks		3 Days		1 Day	
Control 1	1.672E-06	Control 1	9.373E-07	Control 1	1.303E-06
Control 2	1.649E-06	Control 2	1.421E-06	Control 2	1.486E-06
Control 3	8.745E-07	Control 3	3.963E-06	Control 3	3.175E-06
Control 4	6.446E-07	Control 4	1.372E-06	Control 4	4.654E-07
Control 5	8.776E-07	Control 5	8.047E-07	Control 5	6.885E-07
Control 6	1.798E-06	Control 6	2.688E-06	Control 6	6.781E-06
Control 7	5.762E-06	Control 7	1.927E-06	Control 7	2.543E-06
Control 8	2.806E-07	Control 8	1.115E-06	Control 8	1.207E-06
Control 9	1.649E-06	Control 9	1.456E-06	Control 9	1.276E-06
Control 10	7.773E-07	Control 10	7.773E-07	Control 10	1.228E-06
Mean \pm SEM	1.753E-06 \pm 2.688E-07	SD	1.472E-06	% CV	83.959
TEK					
2 Weeks		3 Days		1 Day	
Control 1	2.624E-06	Control 1	2.773E-06	Control 1	2.745E-06
Control 2	1.406E-06	Control 2	5.702E-06	Control 2	4.845E-06
Control 3	1.008E-06	Control 3	6.219E-06	Control 3	3.584E-06
Control 4	7.379E-07	Control 4	3.208E-06	Control 4	7.354E-07
Control 5	4.686E-07	Control 5	2.901E-06	Control 5	1.571E-06
Control 6	2.284E-06	Control 6	3.547E-06	Control 6	1.587E-06
Control 7	1.401E-06	Control 7	4.616E-06	Control 7	1.555E-06
Control 8	1.033E-06	Control 8	1.961E-06	Control 8	2.891E-06
Control 9	1.377E-06	Control 9	4.552E-06	Control 9	1.615E-06
Control 10	2.316E-06	Control 10	8.245E-07	Control 10	1.263E-06
Mean \pm SEM	2.445E-06 \pm 2.793E-07	SD	1.530E-06	% CV	62.578
TGFB1					
2 Weeks		3 Days		1 Day	
Control 1	1.717E-05	Control 1	4.913E-06	Control 1	9.624E-06
Control 2	1.484E-05	Control 2	4.322E-06	Control 2	2.158E-05
Control 3	2.106E-05	Control 3	9.590E-06	Control 3	3.542E-05
Control 4	2.741E-05	Control 4	4.879E-06	Control 4	8.613E-06
Control 5	3.294E-05	Control 5	1.705E-05	Control 5	7.709E-06
Control 6	6.154E-06	Control 6	5.986E-06	Control 6	2.260E-06
Control 7	2.183E-06	Control 7	1.536E-05	Control 7	2.552E-06
Control 8	1.146E-06	Control 8	1.521E-05	Control 8	5.842E-06
Control 9	2.013E-05	Control 9	5.643E-06	Control 9	1.014E-05
Control 10	1.918E-05	Control 10	1.394E-05	Control 10	4.443E-06
Mean \pm SEM	1.224E-05 \pm 1.664E-06	SD	9.113E-06	% CV	74.437

Table 56 Variability of SELE, TEK and TGFB1 mRNA Expression in Control Samples

C_q values were log transformed in order to represent the mean, the standard error of the mean, the standard deviation and the coefficient of variation for all control samples used in the orthodontic experiments.

TGFBR1					
2 Weeks		3 Days		1 Day	
Control 1	5.715E-05	Control 1	1.840E-05	Control 1	4.468E-05
Control 2	5.855E-05	Control 2	3.506E-05	Control 2	1.322E-04
Control 3	4.407E-05	Control 3	4.739E-05	Control 3	9.915E-05
Control 4	4.242E-05	Control 4	3.271E-05	Control 4	5.218E-07
Control 5	5.082E-05	Control 5	3.105E-05	Control 5	6.697E-07
Control 6	4.437E-05	Control 6	5.754E-05	Control 6	4.194E-07
Control 7	2.927E-05	Control 7	6.633E-05	Control 7	9.616E-08
Control 8	2.403E-05	Control 8	7.385E-05	Control 8	5.593E-07
Control 9	3.654E-05	Control 9	9.544E-05	Control 9	3.212E-07
Control 10	2.479E-05	Control 10	7.540E-05	Control 10	7.178E-07
Mean \pm SEM	4.081E-05 \pm 6.017E-06	SD	3.296E-05	% CV	80.756
VEGFA					
2 Weeks		3 Days		1 Day	
Control 1	1.057E-05	Control 1	4.352E-06	Control 1	4.896E-06
Control 2	4.103E-06	Control 2	4.103E-06	Control 2	1.536E-05
Control 3	5.566E-06	Control 3	5.762E-06	Control 3	1.306E-05
Control 4	8.064E-06	Control 4	3.647E-06	Control 4	4.981E-06
Control 5	2.000E-05	Control 5	7.094E-06	Control 5	4.999E-06
Control 6	5.321E-06	Control 6	7.709E-06	Control 6	3.723E-06
Control 7	6.805E-06	Control 7	7.763E-06	Control 7	2.423E-06
Control 8	1.901E-06	Control 8	7.268E-06	Control 8	4.262E-06
Control 9	1.035E-05	Control 9	5.489E-06	Control 9	4.428E-06
Control 10	9.524E-06	Control 10	8.764E-06	Control 10	3.142E-06
Mean \pm SEM	6.847E-06 \pm 7.220E-07	SD	3.995E-06	% CV	57.753

Table 57 Variability of TGFBR1 and VEGFA mRNA Expression in Control Samples

C_q values were log transformed in order to represent the mean, the standard error of the mean, the standard deviation and the coefficient of variation for all control samples used in the orthodontic experiments.

4.4.3 Linking Trends in Biological Replicates to those in RT-qPCR

Data

In order to identify inter- and intra-patient trends in mRNA expression levels, C_q values for each gene in each patient were normalised according to the method for relative expression ratio calculation as outlined by Pfaffl *et al.* (2002). Data was then input into a true/false equation in Microsoft Office Excel® where parameters were set against the difference between the calculated relative expression ratio and the ratio at which there is no change in mRNA expression between treated and control samples (value given as 1). All differences found to be greater than zero were said to be due to an up-regulation in mRNA expression of the GOI in that patient, while all differences found to be less than zero were said to be due to a down-regulation of mRNA expression. Significance was not calculated and trends are shown as a purely qualitative assessment of the data.

One Day	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
ANGPT1	UP	UP	UP	UP	DOWN	DOWN	DOWN	UP	UP	UP
FGF2	DOWN	DOWN	UP	UP	DOWN	UP	UP	UP	UP	DOWN
HIF1A	DOWN	DOWN	UP	UP	DOWN	UP	UP	UP	UP	DOWN
IL8	DOWN	DOWN	DOWN	DOWN	DOWN	UP	UP	DOWN	DOWN	UP
ITGAV	UP	DOWN	UP	UP	UP	DOWN	UP	UP	UP	DOWN
ITGB3	DOWN	DOWN	UP	UP	UP	DOWN	DOWN	DOWN	DOWN	DOWN
KDR	DOWN	DOWN	UP	UP	DOWN	DOWN	DOWN	UP	UP	DOWN
MMP2	DOWN	DOWN	UP	UP	DOWN	UP	UP	DOWN	DOWN	DOWN
NOS2	UP	DOWN	UP	UP	DOWN	DOWN	DOWN	DOWN	UP	DOWN
PDGFA	DOWN	DOWN	UP	UP	DOWN	DOWN	DOWN	UP	UP	DOWN
PDGFB	DOWN	DOWN	UP	DOWN	DOWN	DOWN	DOWN	DOWN	UP	DOWN
ROBO4	DOWN	DOWN	UP	DOWN	DOWN	DOWN	UP	UP	DOWN	DOWN
SELE	UP	UP	UP	DOWN	UP	DOWN	DOWN	UP	DOWN	DOWN
TEK	UP	DOWN	UP	UP	UP	UP	UP	UP	UP	DOWN
TGFB1	UP	DOWN	UP	DOWN	DOWN	UP	DOWN	UP	DOWN	DOWN
TGFBR1	UP	DOWN	UP	DOWN	DOWN	DOWN	UP	DOWN	UP	DOWN
VEGFA	UP	DOWN	UP	DOWN	DOWN	DOWN	UP	UP	UP	DOWN

Table 58 Inter- and intra-patient trends in mRNA expression following 1 day orthodontic force application

Following 1 day of orthodontic force application there appeared to be a trend in the down-regulation of PDGFB mRNA expression and the up-regulation of TEK mRNA

expression among the majority of the patients (n = 8) (**Table 58**). In the remaining GOIs, trends of either up- or down-regulation were weak and could not be attributed to the duration of force application due to the limitations of the sample size analysed. There appeared to be a stronger predisposition toward up- or down-regulation of all GOIs within a single patient. In Patient 2, Patient 5 and Patient 10 there was a strong trend ($\geq 80\%$) toward down-regulated mRNA expression of all GOIs (**Table 58**). In addition to mRNA expression being mutually down-regulated in patients, it was identified that in Patient 2 and Patient 10, a mutual up-regulation of ANGPT1 mRNA expression occurred; while in Patient 2 and Patient 5 a mutual up-regulation of SELE mRNA expression was observed. There were no genes mutually up-regulated in Patients 5 and 10. Patient 3 exhibited up-regulated mRNA expression of all analysed GOIs, with the exception of IL8 (**Table 58**).

Three Days	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
ANGPT1	UP	UP	UP	DOWN	DOWN	DOWN	DOWN	UP	DOWN	UP
FGF2	UP	DOWN	UP	DOWN	DOWN	DOWN	UP	UP	DOWN	UP
HIF1A	UP	UP	UP	UP	DOWN	DOWN	DOWN	UP	UP	DOWN
IL8	UP	UP	UP	DOWN	DOWN	DOWN	UP	DOWN	UP	DOWN
ITGAV	UP	UP	UP	DOWN	DOWN	DOWN	DOWN	DOWN	UP	UP
ITGB3	UP	DOWN	UP	DOWN	DOWN	DOWN	DOWN	UP	UP	DOWN
KDR	DOWN	UP	UP	DOWN	UP	UP	DOWN	DOWN	DOWN	UP
MMP2	UP	UP	UP	DOWN	DOWN	UP	DOWN	UP	DOWN	UP
NOS2	UP	DOWN	UP	DOWN	UP	DOWN	DOWN	UP	DOWN	DOWN
PDGFA	DOWN	DOWN	UP	UP	UP	DOWN	UP	UP	UP	DOWN
PDGFB	UP	UP	UP	DOWN	UP	UP	DOWN	UP	DOWN	UP
ROBO4	UP	UP	DOWN	UP	DOWN	DOWN	DOWN	DOWN	DOWN	UP
SELE	UP	DOWN	UP	DOWN	DOWN	UP	DOWN	UP	DOWN	UP
TEK	DOWN	UP	UP	UP	DOWN	UP	DOWN	UP	UP	UP
TGFB1	UP	DOWN	UP	DOWN	DOWN	UP	DOWN	DOWN	UP	UP
TGFBR1	UP	UP	UP	DOWN	DOWN	DOWN	DOWN	UP	UP	UP
VEGFA	UP	UP	UP	DOWN	DOWN	DOWN	DOWN	UP	DOWN	UP

Table 59 Inter- and intra-patient trends in mRNA expression following 3 days of orthodontic force application

After 3 days of orthodontic force application, no distinct trends in up- or down-regulation of mRNA expression could be identified for any of the GOIs (**Table 59**).

mRNA expression was shown to be up-regulated in approximately 50 % of the sample group, while the other 50 % of the sample group showed a down-regulation of mRNA expression for the same GOI. Upon investigating trends within individual patients, it was shown that Patient 4, Patient 5 and Patient 7 showed a strong trend (≥ 80 %) towards down-regulation of mRNA expression; while Patient 1 and Patient 3 exhibited strong trends (≥ 80 %) toward up-regulation of mRNA expression for the GOIs (**Table 59**). While Patients 4, 5 and 7 exhibited a strong trend (≥ 80 %) toward down-regulated mRNA expression; it was observed that they shared a mutual up-regulation of PDGFA mRNA. The remaining genes found to be up-regulated in these patients were shown to be entirely disparate. Patient 1 and Patient 3 did not share mutual down-regulation of mRNA expression for any of the GOIs (**Table 59**).

Two Weeks	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
ANGPT1	DOWN	UP	UP	UP	DOWN	UP	UP	DOWN	UP	UP
FGF2	DOWN	DOWN	UP	UP	DOWN	UP	UP	DOWN	UP	UP
HIF1A	DOWN	UP	DOWN	UP	UP	UP	UP	UP	DOWN	DOWN
IL8	UP	UP	UP	DOWN	UP	DOWN	UP	DOWN	UP	UP
ITGAV	DOWN	DOWN	DOWN	UP	DOWN	UP	UP	UP	UP	DOWN
ITGB3	UP	DOWN	UP	UP	DOWN	UP	UP	DOWN	UP	UP
KDR	UP	DOWN	DOWN	DOWN	DOWN	UP	UP	DOWN	UP	DOWN
MMP2	UP	UP	DOWN	UP	DOWN	DOWN	UP	DOWN	UP	UP
NOS2	DOWN	DOWN	DOWN	DOWN	DOWN	UP	UP	DOWN	UP	DOWN
PDGFA	DOWN	DOWN	UP	DOWN	DOWN	UP	UP	DOWN	UP	UP
PDGFB	UP	DOWN	DOWN	DOWN	DOWN	UP	UP	DOWN	UP	DOWN
ROBO4	DOWN	DOWN	UP	UP	DOWN	UP	DOWN	DOWN	UP	UP
SELE	DOWN	DOWN	UP	UP	UP	UP	UP	DOWN	UP	DOWN
TEK	DOWN	DOWN	DOWN	DOWN	DOWN	UP	UP	UP	DOWN	DOWN
TGFB1	DOWN	UP	DOWN	UP	DOWN	DOWN	UP	DOWN	UP	DOWN
TGFB1	DOWN	UP	DOWN	DOWN	DOWN	UP	UP	DOWN	UP	DOWN
VEGFA	UP	DOWN	UP	UP	DOWN	UP	UP	DOWN	UP	UP

Table 60 Inter- and intra-patient trends in mRNA expression following 2 weeks (14 days) of orthodontic force application

Following an application of orthodontic force for a period of 2 weeks, there were no distinct trends in up- or down-regulation of mRNA expression in any of the GOIs (**Table 60**). When individual patients were examined, a distinct trend of up-regulated

mRNA expression was observed in Patient 6, Patient 7 and Patient 9; however there were no genes for which a mutual down-regulation of mRNA expression was observed (**Table 60**). Patient 5 and Patient 8 exhibited a trend toward down-regulated mRNA expression, however only HIF1A mRNA was found to be mutually up-regulated in these patients (**Table 60**).

4.4.3.1 *The Effect of Phenotypic Factors on mRNA Expression Levels*

In order to determine whether a patient's phenotypic characteristics (i.e. ethnicity, gender and apical/apex morphology of the tooth from which the pulp was extracted) played a specific role in mRNA expression patterning among the GOIs; patient details (**Table 61**) were compared directly to trends calculated in **Table 58 – Table 60**.

In pulps isolated from teeth treated with an orthodontic force for 1 day prior to extraction, we previously showed a trend in down-regulation of mRNA expression in Patient 2, Patient 5 and Patient 10 (**Table 58**). When analysing the patients' phenotypic characteristics we were able to link all three patients by gender, however this could not be described as the sole contributing factor to the trend in down-regulation as 7 of the 10 patients analysed in this treatment group belonged to the same gender (**Table 61**). Patient 2 and Patient 10 showed a mutual up-regulation of ANGPT1 mRNA expression and shared similar tooth apex morphology in combination with being of the same gender (**Table 61**). Although Patient 2 and Patient 5 were shown to share a mutual up-regulation of SELE mRNA expression, the only phenotype linking the patients was gender (**Table 61**). Patient 5 and Patient 10 shared no mutual up-regulation of any of the GOIs but did share the same ethnicity and gender (**Table 61**).

In patients treated with an orthodontic force for 3 days prior to tooth extraction there was a trend of up-regulated mRNA expression in Patient 1 and Patient 3; and a trend of down-regulated mRNA expression in Patient 4, Patient 5 and Patient 7 (**Table 59**).

When phenotypic characteristics were investigated there was shown to be no linking phenotype between Patient 1 and Patient 3 despite the observed trend in up-regulated mRNA expression. In Patient 4, Patient 5 and Patient 7 it was previously identified that PDGFA mRNA expression was mutually up-regulated (**Table 59**), however there were no phenotypic factors linking all three of these patients. Patient 4 and Patient 5 shared both the same ethnicity and gender while Patient 5 and Patient 7 shared the same apex morphology (**Table 61**).

In the two week treated group Patient 6, Patient 7 and Patient 9 showed a general trend of up-regulated mRNA expression, while Patient 5 and Patient 8 showed a general trend of down-regulated mRNA expression in the GOIs (**Table 60**). Upon analysing the phenotypic characteristics of each of these patients it was noted that Patient 6, Patient 7 and Patient 9 were linked by apex morphology while Patient 5 and Patient 8 were linked only by gender (**Table 61**).

One Day	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
Age	13 Years 7 Months	14 Years 4 Months	11 Years 1 Month	13 Years 8 Months	14 Years 4 Months	13 Years 9 Months	13 Years 3 Months	12 Years 8 Months	12 Years	11 Years
Gender	F	F	M	F	F	F	M	F	F	F
Ethnicity	White	White	Asian	Asian	Black	Asian	Black	Black	White	Black
Apical Morphology	Closed	Almost Closed	Almost Closed	Closed	Closed	Closed	Almost Closed	Closed	Almost Closed	Almost Closed
Three Days	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
Age	10 Years 1 Month	10 Years 3 Months	14 Years 7 Months	13 Years 2 Months	13 Years	12 Years	14 Years 6 Months	15 Years 7 Months	13 Years 8 Months	13 Years 5 Months
Gender	F	F	M	F	F	M	M	F	F	F
Ethnicity	White	Black	Asian	Black	Black	Asian	Chinese	White	Black	Black
Apical Morphology	Open	Open	Closed	Almost Closed	Closed	Almost Closed	Closed	Closed	Closed	Closed
Two Weeks	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
Age	12 Years 5 Months	14 Years 1 Month	14 Years 3 Months	11 Years 5 Months	13 Years 8 Months	11 Years 3 Months	14 Years 4 Months	12 Years	13 Years 9 Months	12 Years 8 Months
Gender	F	M	M	F	F	F	M	F	F	F
Ethnicity	Black	White	White	Asian	Black	White	Black	Asian	Chinese	White
Apical Morphology	Open	Closed	Closed	Open	Closed	Closed	Closed	Almost Closed	Closed	Almost Closed

Table 61 Details of patients' age, gender, ethnicity and root morphology

In order to determine whether age, gender, ethnicity or apex morphology had any significant effect on mRNA expression levels across all time-points of orthodontic force application, C_q values were normalised and a multivariate mixed linear regression analysis was performed (**Chapter 8, section 8.3.1**). It was shown that patient age and gender had no significant effect ($p > 0.05$) on the mRNA expression of any of the GOIs (**Table 62 & Chapter 8, section 8.3.1**). Patient ethnicity had a significant effect ($p \leq 0.05$) on the expression of HIF1A and NOS2 mRNA; and had a borderline significant effect ($0.12 \leq p < 0.05$) on TGFBR1 mRNA expression (**Table 62 & Chapter 8, section 8.3.1**). Apex morphology was shown to have a borderline significant effect ($0.12 \leq p < 0.05$) on IL8 mRNA expression across the three treatment time-points (**Table 62 & Chapter 8, section 8.3.1**).

Angiogenesis-Specific Growth Factor	Age	Gender	Ethnicity	Apex Morphology
ANGPT1	✗	✗	✗	✗
FGF2	✗	✗	✗	✗
HIF1A	✗	✗	✓	✗
IL8	✗	✗	✗	BORDERLINE
ITGAV	✗	✗	✗	✗
ITGB3	✗	✗	✗	✗
KDR	✗	✗	✗	✗
MMP2	✗	✗	✗	✗
NOS2	✗	✗	✓	✗
PDGFA	✗	✗	✗	✗
PDGFB	✗	✗	✗	✗
ROBO4	✗	✗	✗	✗
SELE	✗	✗	✗	✗
TEK	✗	✗	✗	✗
TGFB1	✗	✗	✗	✗
TGFBR1	✗	✗	BORDERLINE	✗
VEGFA	✗	✗	✗	✗

Table 62 Multivariate mixed linear regression analysis identifies the impact of patient phenotype on mRNA expression

Details for multivariate mixed linear regression analyses for individual genes can be found in **Chapter 8, section 8.3.1**

4.4.3.2 *The Effect of Orthodontic Force Duration on mRNA Expression Levels*

Concurrent to the multiple mixed linear regression analysis, a one-way ANOVA (analysis of variance) test was performed in order to determine whether or not the application of orthodontic force for any period of time had a significant effect on gene expression levels in the human dental pulp (**Chapter 8, section 8.3.2**). Subsequently, a pair-wise regression analysis was performed on the data to assess the differences in mRNA expression between each of the treatment groups. Due to the sample size significance was given at ($p \leq 0.05$) and borderline significance was given at ($0.12 \leq p < 0.05$).

It was shown that the application of an orthodontic force for any period of time had a significant effect on the mRNA expression of ANGPT1, FGF2, HIF1A and NOS2; and a borderline significant effect on ITGAV, KDR, PDGFA and TGFBR1 mRNA expression (**Table 63 & Chapter 8, section 8.3.2**).

There was a significant difference in the mRNA expression of ANGPT1 and HIF1A between 1 and 3 days of orthodontic force application; and a borderline significant difference in FGF2, ITGAV and NOS2 mRNA expression at these time-points of orthodontic treatment (**Table 63 & Chapter 8, section 8.3.2**).

Between 1 day and 2 weeks (14 days) of orthodontic force application, there was shown to be a significant difference in the mRNA expression of NOS2; and a borderline significant difference in HIF1A, KDR and TGFBR1 mRNA expression between these treatment time-points (**Table 63 & Chapter 8, section 8.3.2**).

It was shown that between 3 days and 2 weeks of orthodontic force application, there was a significant difference in the expression of ANGPT1, FGF2, HIF1A, ITGAV, KDR, PDGFA, TEK and TGFBR1. There was also a borderline significant difference in the mRNA expression of ITGB3 and MMP2 (**Table 63 & Chapter 8, section 8.3.2**).

Angiogenesis-Specific Growth Factor	Force Application	1 Day vs 3 Days	1 Day vs 14 Days	3 Days vs 14 Days
ANGPT1	✓	✓	✗	✓
FGF2	✓	BORDERLINE	✗	✓
HIF1A	✓	✓	BORDERLINE	✓
IL8	✗	✗	✗	✗
ITGAV	BORDERLINE	BORDERLINE	✗	✓
ITGB3	✗	✗	✗	BORDERLINE
KDR	BORDERLINE	✗	BORDERLINE	✓
MMP2	✗	✗	✗	BORDERLINE
NOS2	✓	BORDERLINE	✓	✗
PDGFA	BORDERLINE	✗	✗	✓
PDGFB	✗	✗	✗	✗
ROBO4	✗	✗	✗	✗
SELE	✗	✗	✗	✗
TEK	✗	✗	✗	✓
TGFB1	✗	✗	✗	✗
TGFBR1	BORDERLINE	✗	BORDERLINE	✓
VEGFA	✗	✗	✗	✗

Table 63 One-way ANOVA and pair-wise regression analysis of the effect of orthodontic force application and the duration of treatment on mRNA expression

Details for ANOVA and pair-wise regression analyses for individual genes can be found in **Chapter 8, section 8.3.2**

4.4.3.3 Identifying Trends in the Variability of mRNA Expression across all Treatment Time-Points

A factor analysis was performed on normalised C_q values in order to identify commonalities in the variability of mRNA expression for the GOIs across all time-points of orthodontic force application. It was identified that MMP2, TGFB1 and VEGFA mRNA expression was most tightly correlated and changed in the same direction (**Table 64**). mRNA expression of ANGPT1, FGF2, HIF1A, ITGAV, ITGB3, KDR, PDGFA, PDGFB, ROBO4, SELE, TEK and TGFBR1 was also found to be tightly correlated but to a lesser degree than MMP2, TGFB1 and VEGFA (**Table 64**).

IL8 and NOS2 mRNA expression was not correlated with any of the other GOIs (Table 64).

Factor Matrix ^a				
	Factor			
	1	2	3	4
ANGPT1	0.15	0.61	0.27	0.51
FGF2	0.19	0.86	0.29	-0.14
HIF1A	0.26	0.87	0.02	-0.03
IL8	0.34	0.07	-0.08	-0.39
ITGAV	0.12	0.81	0.29	0.13
ITGB3	0.48	0.67	0.17	-0.22
KDR	0.13	0.94	-0.18	-0.06
MMP2	0.58	0.44	-0.09	-0.15
NOS2	-0.04	0.26	0.23	0.13
PDGFA	0.30	0.79	-0.33	0.14
PDGFB	0.30	0.56	-0.39	0.26
ROBO4	0.53	0.59	-0.26	0.00
SELE	-0.08	0.57	0.38	0.14
TEK	0.19	0.66	0.22	-0.29
TGFB1	0.99	-0.01	0.00	0.00
TGFBR1	0.13	0.71	0.27	-0.02
VEGFA	0.58	0.39	0.11	0.32

Table 64 Commonality of variability in mRNA expression of angiogenesis-specific GOIs

A factor greater than or equal to 0.55 was considered to be significant when considering the correlated expression of the GOIs. GOIs considered to be tightly correlated are highlighted in green. GOIs considered to be the most tightly correlated to each other in expression amongst all orthodontic treatment groups are highlighted in blue.

4.4.4 Statistical Standardisation of Biological Variability in RT-qPCR

Data

In order to eliminate the effect of biological, inter-patient variation on the average mRNA expression levels as calculated by the REST-2009© software; C_q values were

log-transformed, mean centred and auto-scaled according to the methodology outlined by Willems *et al.* (2008). The methodology was clarified and validated by Dr Nora Donaldson, Statistical Advisor, Dental Institute, King's College London.

Following analysis of C_q values using the methodology described by Willems *et al.* (2008), it was shown that after 1 day of orthodontic force application TEK mRNA expression was significantly up-regulated ($p = 0.01$) and PDGFB mRNA expression was significantly down-regulated ($p = 0.01$) in treated samples relative to their paired, untreated, contralateral controls (**Figure 17**).

Following 3 days of force application there was a statistically significant down-regulation of ANGPT1 ($p < 0.01$), HIF1A ($p < 0.01$), KDR ($p < 0.01$), PDGFA ($p < 0.01$), PDGFB ($p = 0.01$), ROBO4 ($p = 0.01$), TEK ($p < 0.01$) and TGFBR1 ($p < 0.01$) mRNA expression in the pulps of treated teeth relative to their paired, untreated, contralateral controls (**Figure 18**).

There was a statistically significant up-regulation of HIF1A ($p = 0.01$), ITGAV ($p < 0.01$), KDR ($p = 0.01$) and TGFBR1 ($p = 0.01$) mRNA expression in teeth treated with an orthodontic force for a period of 14 days prior to extraction relative to their paired, untreated, contralateral controls (**Figure 19**).

Statistical significance was given as $p \leq 0.05$.

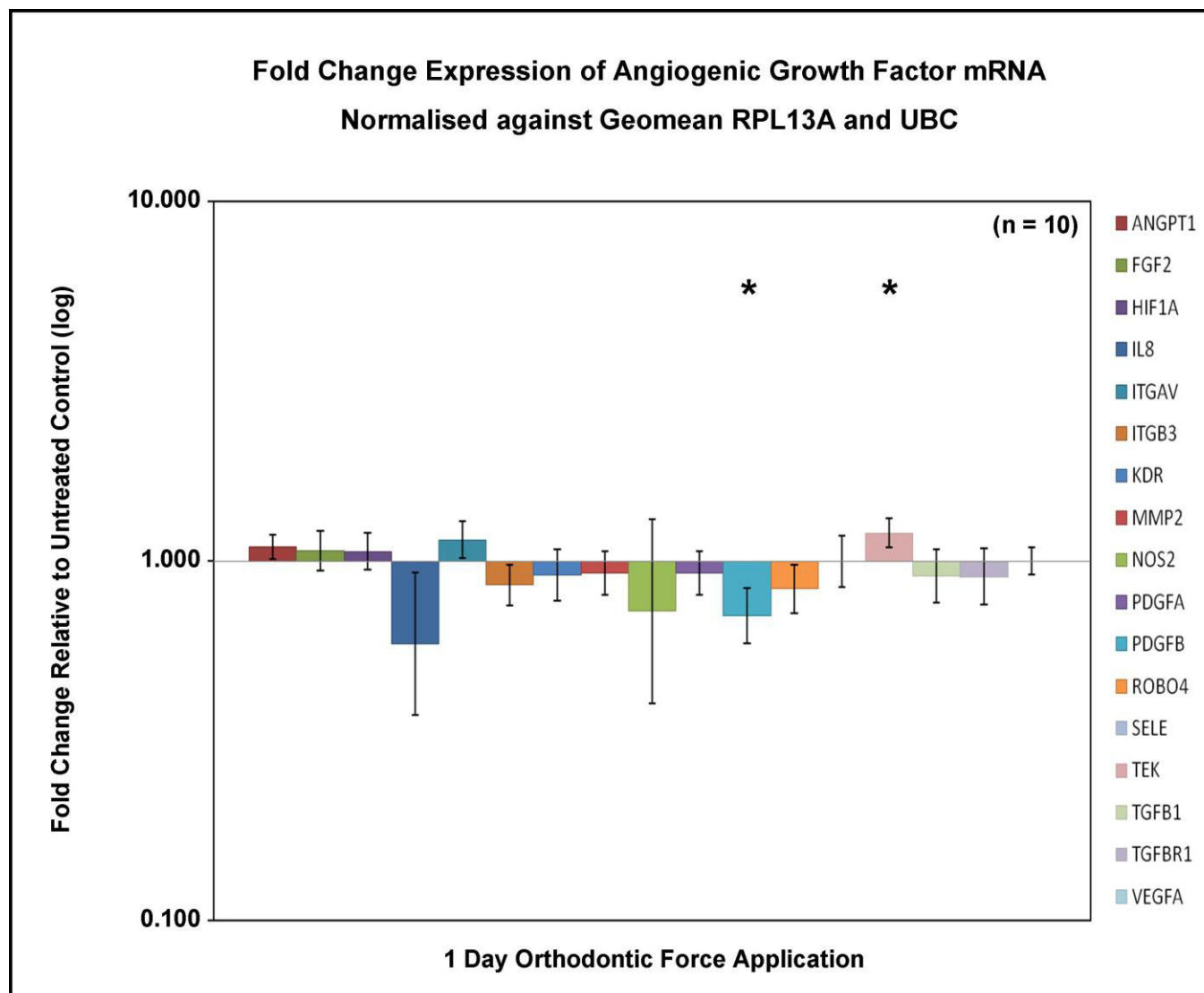


Figure 17 **Fold-change** **expression**
analysis of angiogenic growth factor mRNA in
one day treated samples relative to
contralateral controls. Normalising against
the geometric mean of RPL13A and UBC

Plot shows the fold-change (log) of mRNA
expression (columns); and the error of the mean
(error bars) in one day treated samples (n = 10)
relative to contralateral controls (n = 10). Data
was normalised against the geometric mean of
RPL13A and UBC. There was a significant up-
regulation of TEK mRNA expression and a
significant down-regulation of PDGFB mRNA
expression in treated samples relative to
contralateral controls.

* indicates statistical significance ($p \leq 0.05$).

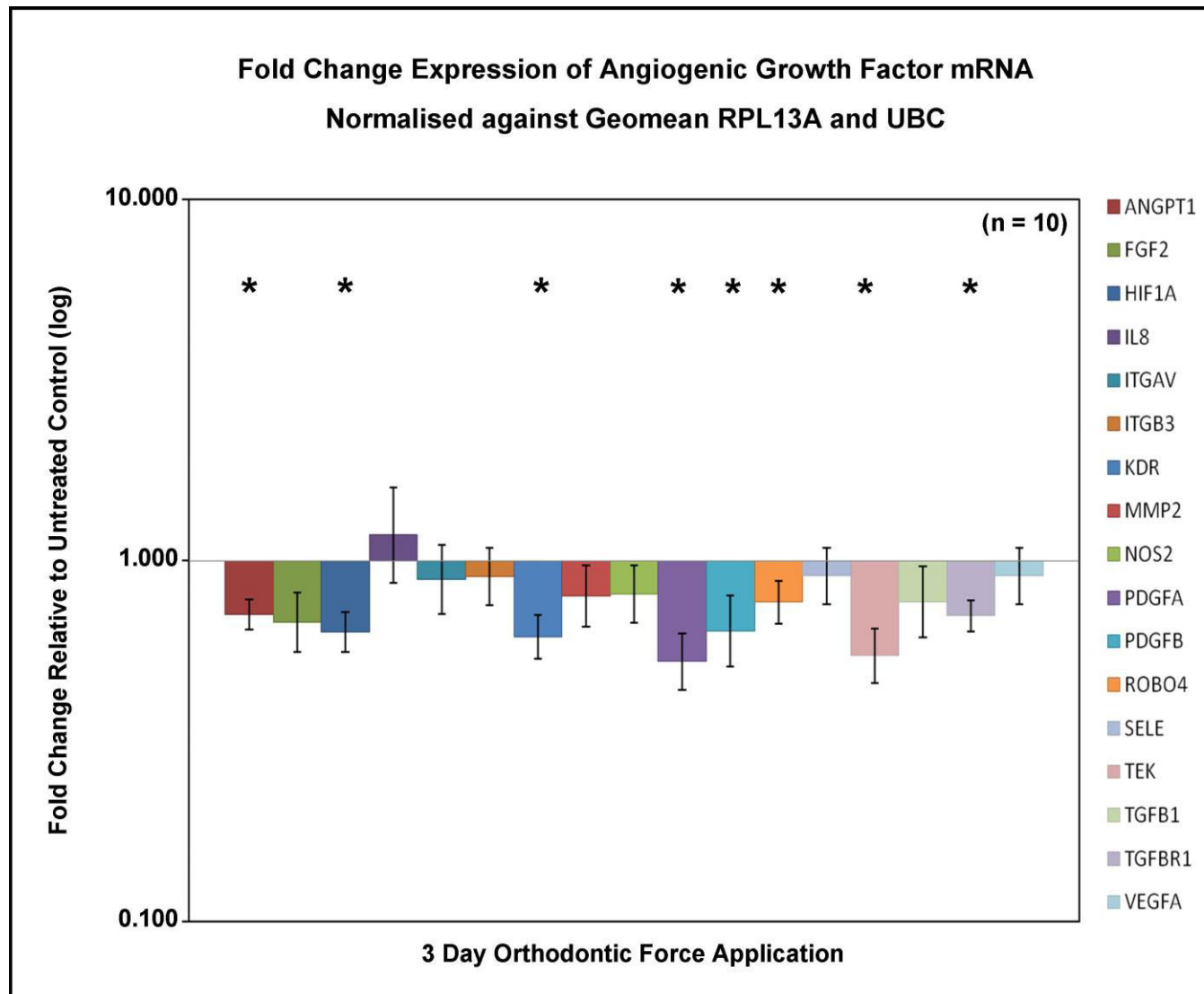


Figure 18 **Fold-change** **expression**
analysis of angiogenic growth factor mRNA in
three day treated samples relative to
contralateral controls. Normalising against
the geometric mean of RPL13A and UBC

Plot shows the fold-change (log) of mRNA
expression (columns); and the error of the mean
(error bars) in three day treated samples (n = 10)
relative to contralateral controls (n = 10). Data
was normalised against the geometric mean of
RPL13A and UBC. There was a significant
down-regulation of ANGPT1, HIF1A, KDR,
PDGFA, PDGFB, ROBO4, TEK and TGFBR1
mRNA expression in treated samples relative to
contralateral controls.

* indicates statistical significance ($p \leq 0.05$).

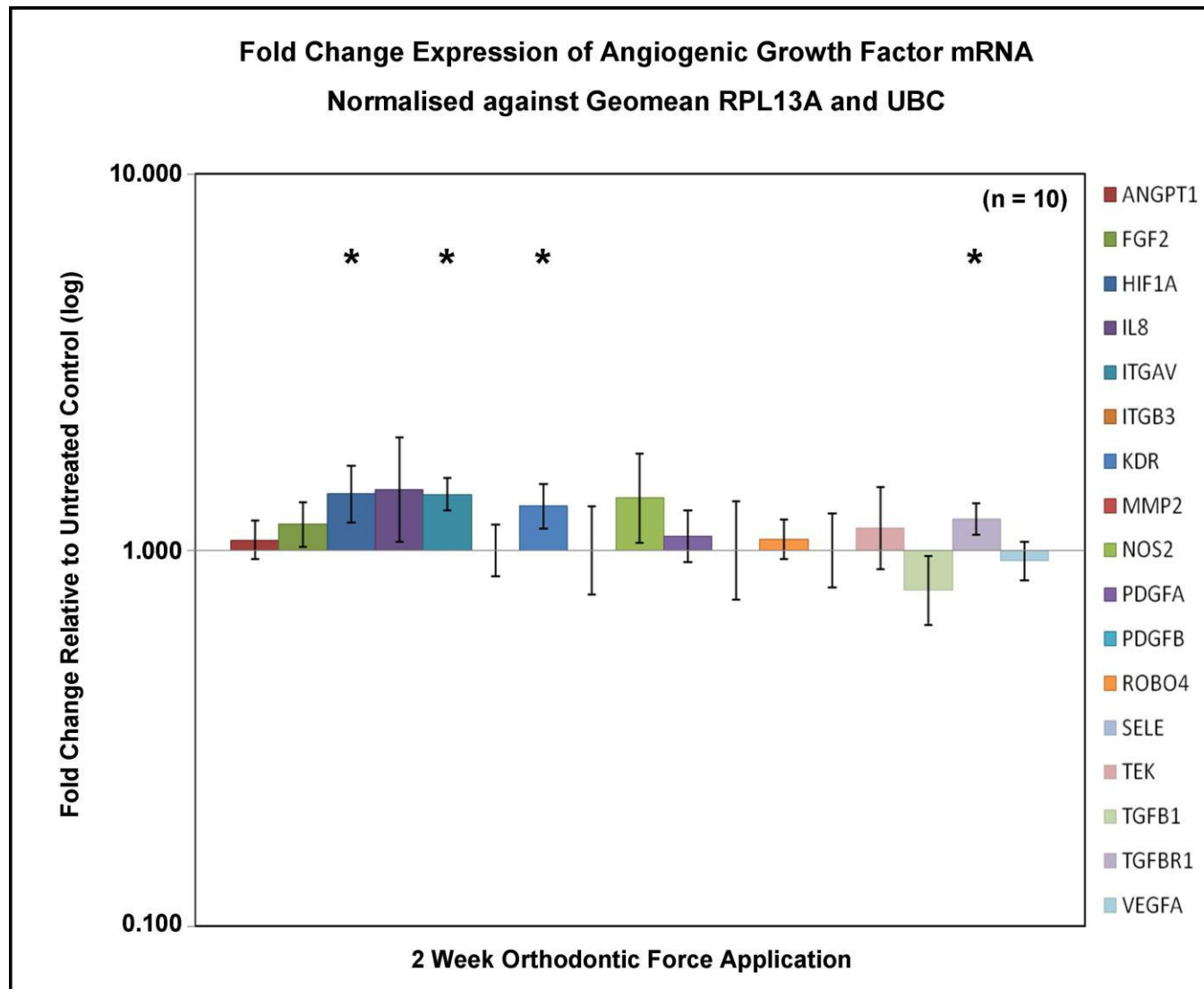


Figure 19 Fold-change expression analysis of angiogenic growth factor mRNA in two week (14 day) treated samples relative to contralateral controls. Normalising against the geometric mean of RPL13A and UBC

Plot shows the fold-change (log) of mRNA expression (columns); and the error of the mean (error bars) in two week treated samples (n = 10) relative to contralateral controls (n = 10). Data was normalised against the geometric mean of RPL13A and UBC. There was a significant up-regulation of HIF1A, ITGAV, KDR and TGFBR1 mRNA expression in treated samples relative to contralateral controls.

* indicates statistical significance ($p \leq 0.05$).

4.4.5 Linking Trends in Protein Expression Profiles to Orthodontic Force Application

In order to identify the effects of orthodontic force application on the protein expression profiles of human dental pulps, a custom multiplex ELISA (Quantibody® Array) was performed by RayBiotech Inc., Georgia, USA on aliquots of human dental pulp lysate at a final concentration of 1 µg/µl.

It was shown that for most of the angiogenesis-specific GOIs analysed, the average level of analyte present in the pulp homogenates was below the assay's lower LOD (Table 65 – Table 67). FGF2 was the only protein of interest in which the concentration of analyte present in the majority of samples under all experimental conditions was greater than the assay's lower LOD (Table 65 – Table 67).

ONE DAY	LIMIT OF DETECTION pg/ml	PATIENT 1		PATIENT 2		PATIENT 3		PATIENT 4		PATIENT 5	
		Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
ANGPT1	105.2	17.8	26.0	45.0	4.3	0.0	0.0	11.8	33.7	0.0	19.1
FGF2	260.2	928.2	753.8	688.4	786.0	488.7	674.6	734.0	982.0	3981.9	1872.1
KDR	19.5	7.8	0.9	0.0	0.8	1.1	0.0	0.0	3.0	4.3	0.0
MMP2	301.9	62.9	71.3	133.3	684.0	0.0	129.7	259.3	0.0	0.0	0.0
PDGFA	23.8	2.0	3.3	21.5	5.2	3.2	0.0	59.6	50.7	0.0	12.2
PDGFB	2.9	0.1	0.0	0.0	0.0	0.0	0.6	0.1	0.2	0.0	0.0
SELE	67.2	75.5	163.1	74.0	71.2	0.0	57.4	27.1	80.7	39.4	43.0
TEK	64.6	70.0	0.0	9.7	25.4	3.1	10.5	30.2	31.9	25.1	0.0
TGFB1	444.0	225.8	6.0	239.9	0.0	0.0	0.0	52.0	153.6	24.4	230.9
VEGFA	7.9	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.6	0.0	1.1

Table 65 Analyte concentrations (pg/ml) of angiogenesis-specific GOIs in human dental pulp explants exposed to one day of orthodontic force application prior to tooth extraction

Cells marked in blue represent the assay's lower LOD (pg/ml). Cells marked in purple represent samples that expressed an analyte concentration greater than the assay's lower LOD. Cells marked in white represent samples which were below the assay's LOD. Cells marked in green represent samples which did not express any detectable concentration of the analyte of interest.

Using values where analyte concentration was greater than the assay's lower LOD in both the control and treated pulps, it was shown that following one day of orthodontic

force application FGF2 expression increased in 3 out of 5 patients. PDGFA was shown to decrease in 1 patient whilst SELE increased in 1 out of 2 patients. As all other results were below the assay's level of detection no comment can be made as to the significance of these results in relation to change in protein expression as a response to force application.

THREE DAYS	LIMIT OF DETECTION pg/ml	PATIENT 1		PATIENT 2		PATIENT 3		PATIENT 4		PATIENT 5	
		Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
ANGPT1	105.2	0.0	21.6	0.0	0.0	30.6	17.4	6.4	25.3	0.0	0.0
FGF2	260.2	1461.6	1187.1	1347.4	728.1	1576.8	2028.9	2369.1	1921.1	961.6	1035.0
KDR	19.5	9.1	6.9	5.6	5.2	4.6	9.2	11.2	19.3	7.5	6.3
MMP2	301.9	0.0	120.8	122.1	0.0	115.5	0.0	103.4	0.0	0.0	288.0
PDGFA	23.8	7.5	6.6	38.6	12.0	1.5	22.1	12.3	19.0	15.4	0.0
PDGFB	2.9	0.0	0.0	0.0	0.0	3.6	6.3	0.0	0.6	0.0	0.0
SELE	67.2	85.1	64.7	155.8	71.0	71.2	138.3	131.4	209.2	193.5	85.7
TEK	64.6	0.0	10.6	66.6	40.0	5.5	51.8	74.3	56.2	86.5	0.0
TGFB1	444.0	411.3	0.0	185.8	21.0	0.0	58.9	301.0	164.4	250.2	0.0
VEGFA	7.9	1.5	0.0	1.6	0.0	1.2	0.0	2.3	3.6	0.0	1.1

Table 66 Analyte concentrations (pg/ml) of angiogenesis-specific GOIs in human dental pulp explants exposed to three days of orthodontic force application prior to tooth extraction

Cells marked in blue represent the assay's lower LOD (pg/ml). Cells marked in purple represent samples that expressed an analyte concentration greater than the assay's lower LOD. Cells marked in white represent samples which were below the assay's LOD. Cells marked in green represent samples which did not express any detectable concentration of the analyte of interest.

Using values where analyte concentration was greater than the assay's lower LOD in both the control and treated pulps, it was shown that following three days of orthodontic force application FGF2 expression decreased in 3 out of 5 patients. PDGFB was shown to increase in 1 patient whilst SELE increased in 2 out of 4 patients. As all other results were below the assay's level of detection no comment can be made as to the significance of these results in relation to change in protein expression as a response to force application.

TWO WEEKS	LIMIT OF DETECTION pg/ml	PATIENT 1		PATIENT 2		PATIENT 3		PATIENT 4		PATIENT 5	
		Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
ANGPT1	105.2	20.3	0.0	0.0	0.0	0.0	0.0	2.0	0.0	0.0	34.6
FGF2	260.2	1125.3	1502.3	1098.7	1329.4	3005.7	3529.3	2659.8	2340.5	1890.7	2098.1
KDR	19.5	4.8	6.1	0.0	0.0	0.5	0.0	6.6	0.3	2.3	8.0
MMP2	301.9	1671.1	43.4	0.0	0.0	0.0	66.5	234.5	0.0	678.1	0.0
PDGFA	23.8	18.6	1.7	0.0	0.0	143.1	84.2	18.0	13.4	14.1	11.2
PDGFB	2.9	1.5	0.0	1.0	8.4	17.0	10.0	7.3	1.0	1.4	0.0
SELE	67.2	69.3	51.1	20.5	65.3	9.2	59.0	73.1	96.2	43.1	67.2
TEK	64.6	10.9	0.0	0.0	0.0	89.8	5.4	28.0	73.5	40.0	25.2
TGFB1	444.0	59.5	0.0	5.2	0.0	999.6	0.0	0.0	0.0	0.0	0.0
VEGFA	7.9	0.8	2.1	0.0	0.0	5.6	0.7	0.0	0.0	0.0	0.0

Table 67 Analyte concentrations (pg/ml) of angiogenesis-specific GOIs in human dental pulp explants exposed to two weeks of orthodontic force application prior to tooth extraction

Cells marked in blue represent the assay's lower LOD (pg/ml). Cells marked in purple represent samples that expressed an analyte concentration greater than the assay's lower LOD. Cells marked in white represent samples which were below the assay's LOD. Cells marked in green represent samples which did not express any detectable concentration of the analyte of interest.

Using values where analyte concentration was greater than the assay's lower LOD in both the control and treated pulps, it was shown that following two weeks of orthodontic force application FGF2 expression increased in 4 out of 5 patients. PDGFA and PDGFB were shown to decrease in 1 patient whilst SELE increased in 1 patient. As all other results were below the assay's level of detection no comment can be made as to the significance of these results in relation to change in protein expression as a response to force application.

4.4.6 Linking Trends in Biological Replicates to Protein Expression Profiles

Further statistical evaluation could not be performed on data from the protein analysis, as calculated analyte concentrations were below the assay LOD and several samples gave a reading of 0 pg/ml. In order to calculate statistical significance, a minimum of 5 pairs of samples from each treatment group is recommended and it was observed that

there were no cases, with the exception of FGF2, in which all 5 sample pairs had an analyte concentration greater than 0 pg/ml for all treatment time-points.

Qualitative analysis was performed in order to identify inter- and intra-patient trends in protein expression profiles. Differences between analyte concentrations (pg/ml) in control and treated pulp explants were calculated and are shown in **Table 68** – **Table 70**. Where one of the values in the pair was 0 pg/ml, this pair was excluded from the analysis. Increased or decreased expression patterns were then compared.

One Day Orthodontic Force Application					
Angiogenesis-Specific Growth Factor Protein (pg/ml)	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
ANGPT1	UP	DOWN	-	UP	-
FGF2	DOWN	UP	UP	UP	DOWN
KDR	DOWN	-	-	-	-
MMP2	UP	UP	-	-	-
PDGFA	UP	DOWN	-	DOWN	-
PDGFB	-	-	-	UP	-
SELE	UP	DOWN	-	UP	UP
TEK	-	UP	UP	UP	-
TGFB1	DOWN	-	-	UP	UP
VEGFA	-	-	-	-	-

Table 68 Inter- and intra-patient trends in protein expression following 1 day of orthodontic force application

In pulps isolated from teeth treated with an orthodontic force for 1 day prior to extraction, there were no strong trends ($\geq 80\%$) toward increased or decreased protein expression for any of the angiogenesis-specific genes or for any of the patients. Weaker trends (60 % – 80 %) of increased protein expression were seen for FGF2, SELE and TEK; and for Patient 4 where there was an increase in protein expression of six of the ten genes analysed (**Table 68**).

Three Day Orthodontic Force Application					
Angiogenesis-Specific Growth Factor Protein (pg/ml)	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
ANGPT1	-	-	DOWN	UP	-
FGF2	DOWN	DOWN	UP	DOWN	UP
KDR	DOWN	DOWN	UP	UP	DOWN
MMP2	-	-	-	-	-
PDGFA	DOWN	DOWN	UP	UP	-
PDGFB	-	-	UP	-	-
SELE	DOWN	DOWN	UP	UP	DOWN
TEK	-	DOWN	UP	DOWN	-
TGFB1	-	DOWN	-	DOWN	-
VEGFA	-	-	-	UP	-

Table 69 Inter- and intra-patients trends in protein expression following 3 days of orthodontic force application

Following an application of orthodontic force for 3 days, there were no strong trends ($\geq 80\%$) in protein expression for any of the patients or for any of the angiogenesis-specific genes analysed. There were weaker trends (60 % – 80 %) observed in two of the patients. Patient 2 showed a weaker trend toward decreased protein expression and Patient 3 showed a weaker trend toward increased protein expression in treated teeth relative to controls. Weaker trends (60 % – 80 %) could be seen for the individual proteins FGF2, KDR and SELE (**Table 69**).

Two Week Orthodontic Force Application					
Angiogenesis-Specific Growth Factor Protein (pg/ml)	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
ANGPT1	-	-	-	-	-
FGF2	UP	UP	UP	DOWN	UP
KDR	UP	-	-	DOWN	UP
MMP2	DOWN	-	-	-	-
PDGFA	DOWN	-	DOWN	DOWN	DOWN
PDGFB	-	UP	DOWN	DOWN	-
SELE	DOWN	UP	UP	UP	UP
TEK	-	-	DOWN	UP	DOWN
TGFB1	-	-	-	-	-
VEGFA	UP	-	DOWN	-	-

Table 70 Inter- and intra-patient trends in protein expression following 2 weeks of orthodontic force application

As a response to orthodontic force application for 2 weeks prior to extraction, strong trends ($\geq 80\%$) were observed in the expression of FGF2, SELE and PDGFA. FGF2 and SELE expression was found to be increased in response to force application, while PDGFA expression was decreased. No weaker trends (60 % – 80 %) in expression were observed for any of the angiogenesis-specific GOIs or for any of the individual patients (Table 70).

4.4.6.1 Linking Patient Phenotypes to Protein Expression Profiles

In order to determine whether phenotypic factors such as ethnicity, gender and apex morphology played a specific role in the observed protein expression profiles; patient details (Table 71) were compared directly to trends identified in Table 68 – Table 70.

One Day	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age	13 Years 3 Months	14 Years 4 Months	13 Years 8 Months	13 Years 9 Months	12 Years 8 Months
Gender	M	F	F	F	F
Ethnicity	Black	Black	Asian	Asian	Black
Apical Morphology	Open	Closed	Closed	Closed	Closed
Three Days	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age	13 Years 3 Months	14 Years 1 Month	13 Years 5 Months	12 Years 1 Month	14 Years 4 Months
Gender	F	F	F	M	F
Ethnicity	Asian	Black	Black	Black	White
Apical Morphology	Open	Closed	Closed	Open	Open
Two Weeks	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age	12 Years 5 Months	14 Years 1 Month	13 Years 8 Months	11 Years 3 Months	12 Years
Gender	F	M	F	F	F
Ethnicity	Black	White	Black	White	Asian
Apical Morphology	Open	Closed	Open	Open	Open

Table 71 Details of patients' age, gender, ethnicity and root morphology in samples for protein analysis

We previously observed no strong trends ($\geq 80\%$) in protein expression in pulps extracted from teeth treated with orthodontic force application for 1 day prior to extraction but observed weaker trends (60 % – 80 %) in FGF2, SELE and TEK protein

expression (**Table 68**). A weaker trend (60 % – 80 %) of increased protein expression was also observed in Patient 4 (**Table 68**). Upon examination of patient phenotype it was noted that two patients shared the phenotype of closed apex morphology, asian ethnicity and female gender (Patient 3 and Patient 4) (**Table 68 & Table 71**); and another two patients shared the phenotype of closed apex morphology, black ethnicity and female gender (Patient 2 and Patient 5) (**Table 68 & Table 71**). In the group with asian ethnicity there was a shared increased expression of FGF2 and TEK protein expression, while in the group with black ethnicity there were no shared trends in protein expression (**Table 68 & Table 71**). The trends previously observed in FGF2, SELE and TEK were not attributable to either one of these phenotypic groups but shared commonalities across the three patient phenotypes.

Following exposure to 3 days of orthodontic force application prior to pulp isolation, there were no strong trends (≥ 80 %) in protein expression relative to the internal controls. Weaker trends (60 % – 80 %) were observed in the expression of FGF2, KDR and SELE; and among Patient 2 and Patient 3 individually (**Table 69**). When patient phenotypes were observed Patient 2 and Patient 3 were found to share the phenotype of closed apex morphology, black ethnicity and female gender (**Table 71**). Although the patient phenotypes matched, it was previously identified that Patient 2 showed a weak trend (60 % – 80 %) towards decreased protein expression while Patient 3 showed a weak trend (60 % – 80 %) toward increased protein expression (**Table 69**). Trends in protein expression in FGF2, KDR and SELE could not be attributed to one form of ethnicity, gender or apex morphology as the trends were observed across a range of phenotypic groups (**Table 69 & Table 71**).

Although it was shown that there were strong trends (≥ 80 %) in expression of FGF2, PDGFA and SELE following 2 weeks of orthodontic force application (**Table 70**); when patient phenotypes were examined there was shown to be no definite phenotypic

cause for these trends (**Table 71**). Patient 1 and Patient 3 shared the phenotype of open apex morphology, black ethnicity and female gender but only shared common trends in FGF2 and PDGFA protein expression (**Table 70 & Table 71**).

4.4.7 Linking mRNA Expression to Protein Expression in Human Dental Pulp

Where possible, experiments for mRNA expression analysis and protein expression analysis were performed on human dental pulps isolated from the same individuals in order to minimise inter-patient biological variability and allow for intra-patient comparisons.

mRNA and protein expression was compared qualitatively as C_q values could only be represented as delta C_q values (as per the normalisation method outlined by Schmittgen *et al.* (2008)) and a number of the protein results had to be excluded as both values in a contralateral-treated pair yielded a protein concentration of 0 pg/ml. No correction for biological variability could be performed on the mRNA data, as the methodology outlined in Willems *et al.* (2008) calls for the mean-centring of data and gives a mean expression ratio in treated samples relative to contralateral controls, thus eliminating the option of observing changes in individuals.

In all treatment time-point groups it was observed that several genes were regulated in the same direction at both the transcriptomic and proteomic levels, although this was not the case for all observations (**Table 72 – Table 74**). Within a single treatment time-point group, the transcriptomic and proteomic expression patterns were not identical amongst all individuals or all GOIs.

In the one day treated group it was previously shown that TEK mRNA was significantly up-regulated whilst PDGFB was significantly down-regulated. The up-regulation of TEK mRNA was matched by an increase in TEK protein expression in 3 of the 5

patients examined at both the transcriptomic and proteomic levels. PDGFB expression was not matched at the transcriptomic and proteomic levels as although all 5 patients examined showed a down-regulation of PDGFB mRNA, only 1 of the 5 patients showed a decrease in PDGFB protein expression. The remaining 4 patients showed a marginal increase or were negative results.

In the three day treated group, only one patient was examined at both the transcriptomic and proteomic levels due to the number of teeth available for extraction from each patient. In this patient the mRNA expression patterns match those previously identified in the biological variability correction where mRNA is significantly down-regulated for ANGPT1, KDR, PDGFA, PDGFB and TEK after 3 days of treatment. The proteomic expression patterns differ in comparison, with only ANGPT1 expression being decreased out of the significantly decreased mRNAs. A number of proteins were increased in this patient in response to treatment, with FGF2, PDGFB and SELE all being above the lower LOD for the assay.

After two weeks of force application there was previously shown to be a statistically significant increase in KDR mRNA expression. This was true in 3 of the 5 patients analysed in the group and only correlated with the proteomic expression of 1 of the 3 patients.

ONE DAY	PATIENT 4			PATIENT 5			PATIENT 6			PATIENT 7			PATIENT 8		
	Control	Treated	Change in Expression	Control	Treated	Change in Expression	Control	Treated	Change in Expression	Control	Treated	Change in Expression	Control	Treated	Change in Expression
ANGPT1	2.99E-01	3.48E-01	UP	4.15E-01	3.96E-01	DOWN	5.60E-01	4.10E-01	DOWN	4.53E-01	4.26E-01	DOWN	1.36E-01	1.38E-01	UP
FGF2	4.95E-02	6.92E-02	UP	6.22E-02	5.23E-02	DOWN	4.81E-02	5.95E-02	UP	3.33E-02	3.74E-02	UP	4.84E-02	6.04E-02	UP
KDR	1.30E-01	1.43E-01	UP	2.01E-01	1.87E-01	DOWN	1.27E-01	1.04E-01	DOWN	2.39E-01	2.03E-01	DOWN	1.87E-01	2.01E-01	UP
MMP2	4.61E-02	5.47E-02	UP	5.86E-02	4.34E-02	DOWN	2.26E-02	3.21E-02	UP	1.61E-02	2.49E-02	UP	3.20E-02	3.01E-02	DOWN
PDGFA	5.62E-03	7.05E-03	UP	1.02E-02	8.71E-03	DOWN	7.64E-03	5.92E-03	DOWN	8.16E-03	6.29E-03	DOWN	6.12E-03	7.84E-03	UP
PDGFB	3.14E-03	2.08E-03	DOWN	4.36E-03	1.76E-03	DOWN	2.08E-03	8.13E-04	DOWN	1.11E-02	6.14E-03	DOWN	4.11E-03	2.92E-03	DOWN
SELE	1.69E-02	1.61E-02	DOWN	2.09E-02	4.57E-02	UP	1.87E-01	7.54E-02	DOWN	9.26E-02	8.15E-02	DOWN	3.26E-02	5.11E-02	UP
TEK	2.66E-02	2.86E-02	UP	4.78E-02	5.39E-02	UP	4.37E-02	4.95E-02	UP	5.66E-02	6.57E-02	UP	7.82E-02	8.45E-02	UP
TGFB1	3.12E-01	2.59E-01	DOWN	2.34E-01	1.49E-01	DOWN	6.22E-02	1.27E-01	UP	9.29E-02	7.92E-02	DOWN	1.58E-01	2.29E-01	UP
VEGFA	1.80E-01	1.68E-01	DOWN	1.52E-01	1.30E-01	DOWN	1.02E-01	9.74E-02	DOWN	8.82E-02	1.14E-01	UP	1.15E-01	1.29E-01	UP

ONE DAY	PATIENT 3			PATIENT 2			PATIENT 4			PATIENT 1			PATIENT 5		
	Control	Treated	Change in Expression	Control	Treated	Change in Expression	Control	Treated	Change in Expression	Control	Treated	Change in Expression	Control	Treated	Change in Expression
ANGPT1	0.0	0.0	-	45.0	4.3	DOWN	11.8	33.7	UP	17.8	26.0	UP	0.0	19.1	UP
FGF2	488.7	674.6	UP	688.4	786.0	UP	734.0	982.0	UP	928.2	753.8	DOWN	3981.9	1872.1	DOWN
KDR	1.1	0.0	DOWN	0.0	0.8	UP	0.0	3.0	UP	7.8	0.9	DOWN	4.3	0.0	DOWN
MMP2	0.0	129.7	UP	133.3	684.0	UP	259.3	0.0	DOWN	62.9	71.3	UP	0.0	0.0	-
PDGFA	3.2	0.0	DOWN	21.5	5.2	DOWN	59.6	50.7	DOWN	2.0	3.3	UP	0.0	12.2	UP
PDGFB	0.0	0.6	UP	0.0	0.0	-	0.1	0.2	UP	0.1	0.0	DOWN	0.0	0.0	-
SELE	0.0	57.4	UP	74.0	71.2	DOWN	27.1	80.7	UP	75.5	163.1	UP	39.4	43.0	UP
TEK	3.1	10.5	UP	9.7	25.4	UP	30.2	31.9	UP	70.0	0.0	DOWN	25.1	0.0	DOWN
TGFB1	0.0	0.0	-	239.9	0.0	DOWN	52.0	153.6	UP	225.8	6.0	DOWN	24.4	230.9	UP
VEGFA	0.0	0.0	-	1.0	0.0	DOWN	0.0	0.6	UP	0.0	0.0	-	0.0	1.1	UP

Table 72 Comparison of mRNA and protein expression profiles in patients treated with orthodontic force for 1 day prior to tooth extraction

The top block represents the log transformed C_q values obtained from RT-qPCR. Data was normalised against the geometric mean expression of RPL13A and UBC. The bottom block shows data obtained from the custom Quantibody® ELISA array. Change in expression was calculated by subtracting control values from treated values to identify up- / down-regulation of mRNA or increased / decreased protein expression. Patient numbers are provided as per the samples used earlier in this chapter. Prior to protein analysis, patients were randomly assigned to the mRNA study as Patient 1 – 10 for each treatment group. Upon protein analysis, another contralateral pair of pulps was extracted from some patients from the alternate row of dentition (e.g. mRNA from pulps from maxilla, protein pulps from mandible and vice versa). Therefore, Patient 4 from the mRNA expression analysis is paired with Patient 3 from the protein expression analysis (and so forth), as these are pulps which were extracted from the same individual. Cells marked as pink show a common trend in mRNA and protein expression in individual patients

THREE DAYS	PATIENT 10		
	Control	Treated	Change in Expression
ANGPT1	6.66E+00	1.46E+00	DOWN
FGF2	1.25E+00	4.79E-01	DOWN
KDR	2.95E+00	3.19E-01	DOWN
MMP2	4.80E-01	1.76E-01	DOWN
PDGFA	1.51E-01	2.25E-03	DOWN
PDGFB	3.51E-01	2.12E-02	DOWN
SELE	1.58E-01	1.01E-01	DOWN
TEK	1.68E-01	4.20E-02	DOWN
TGFB1	2.84E+00	8.78E-01	DOWN
VEGFA	1.78E+00	5.26E-01	DOWN
THREE DAYS	PATIENT 3		
	Control	Treated	Change in Expression
ANGPT1	30.6	17.4	DOWN
FGF2	1576.8	2028.9	UP
KDR	4.6	9.2	UP
MMP2	115.5	0.0	DOWN
PDGFA	1.5	22.1	UP
PDGFB	3.6	6.3	UP
SELE	71.2	138.3	UP
TEK	5.5	51.8	UP
TGFB1	0.0	58.9	UP
VEGFA	1.2	0.0	DOWN

Table 73 Comparison of mRNA and protein expression profiles in patients treated with orthodontic force for 3 days prior to tooth extraction

The top block represents the log transformed C_q values obtained from RT-qPCR. Data was normalised against the geometric mean expression of RPL13A and UBC. The bottom block shows data obtained from the custom Quantibody® ELISA array. Change in expression was calculated by subtracting control values from treated values to identify up- / down-regulation of mRNA or increased / decreased protein expression. Patient numbers are provided as per the samples used earlier in this chapter. Prior to protein analysis, patients were randomly assigned to the mRNA study as Patient 1 – 10 for each treatment group. Upon protein analysis, another contralateral pair of pulps was extracted from some patients from the alternate row of dentition (e.g. mRNA from pulps from maxilla, protein pulps from mandible and vice versa). Therefore, Patient 10 from the mRNA expression analysis is paired with Patient 3 from the protein expression analysis, as these are pulps which were extracted from the same individual. Cells marked as pink show a common trend in mRNA and protein expression in individual patients.

TWO WEEKS	PATIENT 1			PATIENT 2			PATIENT 5			PATIENT 6			PATIENT 8		
	Control	Treated	Change in Expression	Control	Treated	Change in Expression	Control	Treated	Change in Expression	Control	Treated	Change in Expression	Control	Treated	Change in Expression
ANGPT1	2.78E-01	4.07E-01	UP	2.41E-01	2.01E-01	DOWN	7.84E-02	8.26E-02	UP	5.61E-01	3.97E-01	DOWN	2.00E-01	3.00E-01	UP
FGF2	4.18E-02	4.93E-02	UP	5.20E-02	4.99E-02	DOWN	5.96E-02	5.49E-02	DOWN	6.06E-02	4.68E-02	DOWN	3.83E-02	4.57E-02	UP
KDR	7.43E-02	1.27E-01	UP	9.12E-02	6.38E-02	DOWN	9.69E-02	9.79E-02	UP	1.60E-01	1.96E-01	UP	7.07E-02	5.55E-02	DOWN
MMP2	1.53E-02	4.43E-02	UP	1.79E-02	7.52E-03	DOWN	4.55E-02	1.10E-02	DOWN	4.25E-02	3.60E-02	DOWN	2.56E-02	2.88E-02	UP
PDGFA	3.67E-03	4.92E-03	UP	4.07E-03	2.91E-03	DOWN	1.02E-02	5.27E-03	DOWN	6.64E-03	1.30E-02	UP	6.08E-03	5.79E-03	DOWN
PDGFB	1.50E-03	1.00E-03	DOWN	1.53E-03	6.72E-04	DOWN	2.61E-03	1.03E-03	DOWN	3.15E-03	5.09E-03	UP	1.44E-03	9.92E-04	DOWN
SELE	1.75E-02	1.11E-02	DOWN	1.91E-02	9.19E-03	DOWN	1.42E-02	1.11E-02	DOWN	4.64E-02	3.45E-02	DOWN	8.65E-03	1.03E-02	UP
TEK	2.75E-02	3.72E-02	UP	1.63E-02	1.18E-02	DOWN	7.56E-03	2.81E-02	UP	5.89E-02	5.17E-02	DOWN	3.19E-02	2.73E-02	DOWN
TGFB1	1.80E-01	1.14E-01	DOWN	1.72E-01	7.99E-02	DOWN	5.31E-01	1.15E-01	DOWN	1.59E-01	2.16E-01	UP	3.53E-02	7.19E-02	UP
VEGFA	1.11E-01	1.05E-01	DOWN	4.75E-02	4.48E-02	DOWN	3.23E-01	6.80E-02	DOWN	1.37E-01	1.25E-01	DOWN	5.86E-02	5.92E-02	UP

TWO WEEKS	PATIENT 1			PATIENT 2			PATIENT 3			PATIENT 4			PATIENT 5		
	Control	Treated	Change in Expression	Control	Treated	Change in Expression	Control	Treated	Change in Expression	Control	Treated	Change in Expression	Control	Treated	Change in Expression
ANGPT1	20.3	0.0	DOWN	0.0	0.0	-	0.0	0.0	-	2.0	0.0	DOWN	0.0	34.6	UP
FGF2	1125.3	1502.3	UP	1098.7	1329.4	UP	3005.7	3529.3	UP	2659.8	2340.5	DOWN	1890.7	2098.1	UP
KDR	4.8	6.1	UP	0.0	0.0	-	0.5	0.0	DOWN	6.6	0.3	DOWN	2.3	8.0	UP
MMP2	1671.1	43.4	DOWN	0.0	0.0	-	0.0	66.5	UP	234.5	0.0	DOWN	678.1	0.0	DOWN
PDGFA	18.6	1.7	DOWN	0.0	0.0	-	143.1	84.2	DOWN	18.0	13.4	DOWN	14.1	11.2	DOWN
PDGFB	1.5	0.0	DOWN	1.0	8.4	UP	17.0	10.0	DOWN	7.3	1.0	DOWN	1.4	0.0	DOWN
SELE	69.3	51.1	DOWN	20.5	65.3	UP	9.2	59.0	UP	73.1	96.2	UP	43.1	67.2	UP
TEK	10.9	0.0	DOWN	0.0	0.0	-	89.8	5.4	DOWN	28.0	73.5	UP	40.0	25.2	DOWN
TGFB1	59.5	0.0	DOWN	5.2	0.0	DOWN	999.6	0.0	DOWN	0.0	0.0	-	0.0	0.0	-
VEGFA	0.8	2.1	UP	0.0	0.0	-	5.6	0.7	DOWN	0.0	0.0	-	0.0	0.0	-

Table 74 Comparison of mRNA and protein expression profiles in patients treated with orthodontic force for 2 weeks prior to tooth extraction

The top block represents the log transformed C_q values obtained from RT-qPCR. Data was normalised against the geometric mean expression of RPL13A and UBC. The bottom block shows data obtained from the custom Quantibody® ELISA array. Change in expression was calculated by subtracting control values from treated values to identify up- / down-regulation of mRNA or increased / decreased protein expression. Prior to protein analysis, patients were randomly assigned to the mRNA study as Patient 1 – 10 for each treatment group. Upon protein analysis, another contralateral pair of pulps was extracted from some patients from the alternate row of dentition (e.g. mRNA from pulps from maxilla, protein pulps from mandible and vice versa). Therefore, Patient 1 from the mRNA expression analysis is paired with Patient 1 from the protein expression analysis (and so forth), as these are pulps which were extracted from the same individual. Cells marked as pink show a common trend in mRNA and protein expression in individual patients.

4.5 Discussion

Previous investigations considering the response of the human dental pulp to orthodontic force application have been carried out in order to determine the true effect of routine orthodontic treatment on the tightly encapsulated loose connective tissue (Abdul Wahab *et al.* 2012, Derringer *et al.* 1996, Hamersky *et al.* 1980, Javed *et al.* 2015, Lazzaretti *et al.* 2014, Mostafa *et al.* 1991, Sano *et al.* 2002, Santamaria *et al.* 2006, Stenvik and Mjor 1970, Subay *et al.* 2001, von Bohl *et al.* 2012, Yamaguchi 2007). It has been determined that routine orthodontic treatment results in the pulp's release of several angiogenic growth factors and cytokines (Derringer *et al.* 1996, Derringer and Linden 2003, Derringer and Linden 2004, Derringer and Linden 2007). The cause of this release of growth factors and the extent to which neovascularisation or possible reparative angiogenesis occurs remains undetermined.

This experimental chapter has focussed on expanding previous assessments in the field by analysing the relative expression of seventeen genes thought to be associated with angiogenic signalling cascades. Analyses were performed on human dental pulps that had been exposed to three separate early time-points of orthodontic treatment prior to tooth extraction. mRNA and protein expression was analysed to determine changes in expression at each of the treatment time-points. As it was previously established that the maturity of the apical foramina plays a potentially significant role in the pulp's response to treatment (Hamersky *et al.* 1980, Mostafa *et al.* 1991), patient phenotypes were examined and compared. Changes in mRNA expression as a response to treatment were then compared to differences in protein expression amongst the individuals, in order to determine if a true pattern of up- or down-regulation of the GOIs existed in treated teeth.

4.5.1 Validating the use of Commercially Purchased Primers

The optimisation of experimental conditions, in order to ascertain the most realistic representation of the physiological response, is a critical step in any *in vivo* experiment. The fundamental importance of normalising experimental RT-qPCR data with an appropriate statistical method, by using the correct combination of reference genes, was previously considered (**Chapter 3**) in order to eliminate any spurious interpretations. Efficiency correction was highlighted as a critical step in this normalisation protocol, as it eliminates any under- or overestimation of gene expression in the samples of interest (Freeman *et al.* 1999, Peirson *et al.* 2003, Pfaffl *et al.* 2002). In this light, we purchased sets of primers from a commercial distributor that designed, optimised and guaranteed the reaction efficiency of all experiments at 100 % in order to eliminate our need for standard curve creation and additional experimental optimisation. This was considered to be the most appropriate course of action as we have expanded our investigation from a panel of four GOIs to seventeen. The financial and time limitations of designing and optimising thirteen additional primers were considered too great and were outweighed by the guarantee of 100 % reaction efficiency.

4.5.2 Relative Expression Analysis Showed Minimal Differences in mRNA Expression at any Treatment Time-Point

It was shown that by analysing raw C_q data using the REST-2009© software package (developed by Prof. M. Pfaffl, Technical University Munich, Germany and Qiagen Ltd., Crawley, UK; <http://www.gene-quantification.com/rest-2009.html>), despite optimising all normalisation protocols, that there were little to no statistically significant changes in the mRNA expression levels of these seventeen GOIs at any of the early time-points of orthodontic treatment investigated. These results were in contrast to studies that have investigated changes in the protein expression of angiogenic growth factors in human

dental pulps of teeth undergoing orthodontic treatment (Derringer *et al.* 1996, Derringer and Linden 1998, Derringer and Linden 2003, Derringer and Linden 2004, Derringer and Linden 2007).

As the previous studies showed an increase in secreted proteins and this can only be attributed to an initial up-regulation of mRNA expression at some point between 1 day and 14 days of force application, it was hypothesised that there must be an underlying factor skewing the data and prohibiting the true changes in mRNA expression from being identified. In order to confirm primer specificity and rule out any alternate binding products, primer sequences were analysed using *in silico* BLAST analysis and several possible alternate genes were identified (**Chapter 8, section 8.1.5.4**). Melt curves (**Chapter 8, section 8.1.6.3**) and gel electrophoretograms (**Chapter 8, section 8.1.7.3**) were then analysed in order to determine the incidence of spurious binding products, primer-dimers or amplification of alternate transcripts which may mask true mRNA expression changes in response to treatment. It was established, that despite primer sequences being specific for several alternate genes, the amplicon sizes were specific to our GOIs and the absence of distinct, observable differences in gene expression between the control and treated pulps could not be attributed to non-specific binding of the primers.

4.5.3 Biological Variability May Play a Significant Role in RT-qPCR mRNA Expression Data Analysis

As alternate amplification products were eliminated as the possible cause of ambiguity in our results, control samples were investigated for variability by analysing the mean gene expression, the standard deviation and the coefficient of variance for each GOI. It was shown that amongst 30 untreated samples, using 10 control samples from each treatment time-point, there was a large amount of variation in the expression of each

GOI amongst the untreated sample group (**Table 52 – Table 57**). This difference in mRNA expression amongst the untreated samples was unexpected as it was hypothesised prior to investigation that a random selection of samples from a small range of ages, genders, ethnicities and root morphologies would drastically reduce or eliminate inter-patient variability. Inconsistencies in the mRNA expression of the GOIs within the untreated samples may possibly be attributable to the cellular composition of individual tissues or the maturity of each tooth; and the lack of homogeneity amongst samples has been noted to be a contributor to erroneous interpretation of RT-qPCR data (Freeman *et al.* 1999). As relative expression analysis calculates the mean mRNA expression of a gene in the control samples and compares the mean difference in treated samples against this baseline, it is critical to note that a large amount of variability between the control samples will radically alter the perceived response to treatment. We can conclude at this stage that biological variability has a substantial effect within our experimental model and must be corrected for prior to performing relative expression analysis.

4.5.4 Investigating Changes within Individuals to Determine the Effect of Force Application on mRNA Expression of Angiogenesis-Specific Genes

At this stage of investigation, no further correction for biological variability was available to the author and qualitative assessment was performed on normalised, individual C_q values as per Pfaffl *et al.* (2002) in collaboration with Dr Nora Donaldson in order to determine whether or not there were trends in the expression of a particular GOI at any of the treatment time-points. The mRNA expression trends within individual patients were also compared. It was shown that following 1 day of orthodontic force application there was a trend toward down-regulation of mRNA amongst Patients 2, 5

and 10, although none of the individual GOIs were expressed in a similar manner amongst all three patients. In Patient 3 there was a trend towards up-regulation of mRNA for all GOIs with the exception of IL8. PDGFB was down-regulated in most patients in this treatment group whilst TEK was up-regulated. Following 3 days of force application there were no direct trends in up- or down-regulation for any of the GOIs. Patients 1 and 3 exhibited a trend towards up-regulation of mRNA expression although they had no gene expression in common. Patients 4, 5 and 7 showed a trend toward down-regulated mRNA expression, yet the only gene expressed in a similar manner amongst all three patients was PDGFA, which was up-regulated. After 2 weeks of orthodontic treatment there were no distinct trends in the mRNA expression of any of the individual GOIs. Patients 6, 7 and 9 showed a general trend toward up-regulation of mRNA expression yet they had no common genes expressed in a similar manner among them. Patients 5 and 8 showed a trend towards down-regulation of mRNA expression but the only gene commonly expressed between the two patients was HIF1A which was up-regulated.

There was no evidence of a conclusive trend in gene expression for any of the GOIs at any experimental treatment time-point amongst the samples analysed. We had hypothesised that by analysing paired C_q values from individuals and identifying differences within each gene, we would be able to recognise any trends previously unobservable when analysing the samples as a group and calculating a mean expression change as per REST analysis. If any trends within the treatment time-points were present as a true response to orthodontic force application for a measured duration, we were unable to identify these changes at this time due to the wide range of variability amongst the untreated 'control' sample group. There was no baseline level against which neither comparisons nor patterns could be determined.

4.5.5 Linking Biological Variability in Control Samples to Phenotypic Factors

In order to assess the data further and attempt to determine if the biological variability seen within the untreated 'control' group was attributable to phenotypic factors such as age, gender, ethnicity or root morphology; a qualitative assessment was performed on the normalised C_q values in collaboration with Dr Nora Donaldson. It was previously shown that following 1 day of orthodontic tooth movement, Patients 2, 5 and 10 exhibited a similar trend towards the down-regulation of mRNA expression. Upon examination of patient phenotypes, the only phenotype linking all three patients was gender. Seven of the ten patients within this group were of the same gender as these patients; therefore we could find no phenotypic cause for the trend towards down-regulation of mRNA within these patients. This was also true in the 3 day treated group where Patients 1 & 3 and Patients 4, 5 and 7 appeared to be linked in expression but no common phenotype could be identified. Following 2 weeks of orthodontic force application Patients 6, 7 and 9 which exhibited a trend towards up-regulation of mRNA expression were linked by apex morphology while Patients 5 and 8 which showed a trend of down-regulated mRNA expression were linked by gender.

The only phenotypic factor which seems noteworthy amongst all treatment time-points is that of apex morphology which was shown to be a common factor in Patients 6, 7 and 9 in the 2 week treated group. Apex morphology has previously been associated with differing responses of the pulp to orthodontic treatment; where teeth with open apices recover more quickly from any detrimental effects of treatment than those with closed apices (Hamersky *et al.* 1980, Mostafa *et al.* 1991). Patients were chosen in the age range where premolars were fully erupted and roots were completely formed (Mostafa *et al.* 1991) and when these patients were examined radiographically, as per routine clinical protocol prior to orthodontic treatment, we observed that the apical foramina of

these teeth were closed. When all samples within the 2 week treatment group were compared and the apical morphology of all patients was assessed, it was observed that six of the ten patients within this treatment group had the same apex morphology. Therefore, while apex morphology has been shown to have a significant effect on the pulp's recovery from orthodontic force application in previous studies, we cannot conclusively ascribe changes of mRNA expression in these samples to a response pattern due to tooth maturity.

In order to substantiate our findings regarding the effect of patient phenotype on gene expression, a multivariate mixed linear regression analysis was performed on the normalised C_q data by Dr Nora Donaldson. Following analysis of all samples across all time-points there was shown to be no statistical significance of the patient's age or gender on the mRNA expression of any of the GOIs. This finding was not expected as it has been highlighted by AlQahtani *et al.* (2010) that females will precede males in tooth development, particularly amongst patients within the same age range as those used in our sample groups. We have no explanation for our findings and propose that the lack of significance observed within our sample group, in relation to gender, is due to the uneven sampling of patients from each gender; and the size of the group itself. We postulate that a large sample group containing an equal number of male and female patients may result in a significantly different mRNA expression pattern between patient genders, due to the maturity of the teeth in each group.

As tooth morphology has previously been directly linked to the patient's age (Mostafa *et al.* 1991, Ramazanzadeh *et al.* 2009), we had assumed that the findings of the statistical analyses of tooth apex morphology on mRNA expression would be corroborative, however this was not found to be true in this instance. This finding further highlights those established by AlQahtani *et al.* (2010) where patient age and tooth morphology have been shown to be linked to gender. As our patient groups were

comprised of a mix of male and female samples, where female pulps are considered to be more mature; the analyses of tooth morphology appears to be skewed by the addition of sample information from the opposing gender. We propose that in order to determine whether patient age or apex morphology holds statistical significance within our experimental group, that data from males and females should be analysed separately using a larger sample group.

Apex morphology of the tooth was shown to have a borderline (5 % – 12 %) significant effect on the mRNA expression of the gene IL8 in the human dental pulp. IL8 is a pro-inflammatory chemokine and has been shown to be present in the human dental pulp solely under inflammatory conditions (Hahn and Liewehr 2007, Huang *et al.* 1999, Silva *et al.* 2009). It has been localised in odontoblasts, lymphocytes, macrophages and endothelial cells by immuno-histochemistry (Hahn and Liewehr 2007) but has not been localised to a specific region within the pulp tissue. The effect of apex morphology on orthodontically treated teeth is an interesting point for consideration as it has already been noted that pulps within teeth with closed apices recover from trauma at a slower rate (Hamersky *et al.* 1980, Mostafa *et al.* 1991). Should orthodontic force application induce an inflammatory response, up-regulating the expression of IL8 and other pro-inflammatory cytokines and chemokines, a tooth with a completed apical morphology would maintain the physiological response for a greater length of time. This could have a negative or potentially deleterious effect on the pulp and should be considered in further future studies.

Surprisingly, patient ethnicity was shown to have a significant effect on the expression of the genes HIF1A and NOS2. It was also shown to have a borderline significant effect on the mRNA expression of the gene TGFBR1. Whilst there are no publications in the literature that focus on the differences in expression of these three specific genes between ethnicities, it is now recognised in the literature that gene expression levels do

differ amongst cell types within an individual, among individuals and between ethnic groups (Spielman *et al.* 2007). As HIF1A and NOS2 continue to hold the focus of researchers as potential biomarkers for high profile population-wide diseases such as Huntington's, cancer, tuberculosis and cystic fibrosis (Anam *et al.* 2015, Borovecki *et al.* 2005, Grasemann *et al.* 2000, McCluskie *et al.* 2004), it is the opinion of the author that the findings identified within this study, that patients of differing ethnicities respond differently to treatment, will be verified and clarified in future research.

4.5.6 The Effect of Force Application on mRNA Expression: A Regression Analysis

Despite the inability to correct for biological variability amongst the untreated control samples, a multivariate mixed linear regression analysis was performed concurrently with a one-way ANOVA (analysis of variance) test in order to identify if any level of force application would have a significant effect on the mRNA expression of the GOIs. Pair-wise regression analyses were then performed on the data for each treatment time-point in order to determine if force application between two specific time-points would result in a significant difference in mRNA expression. The analyses were performed by Dr Nora Donaldson on the normalised C_q values and statistical significance was given at 5 % whilst borderline significance was given from 5 % to 12 %. It was shown that the application of any duration of orthodontic force has a significant effect on the mRNA expression of the genes ANGPT1, FGF2, HIF1A and NOS2. Orthodontic treatment also has a borderline significance on the expression of ITGAV, KDR, PDGFA and TGFBR1.

Using STRING (www.string-db.org), an online database of predicted protein-protein interactions, and the Kyoto Encyclopaedia of Genes and Genomes (KEGG) (<http://www.genome.jp/kegg/>), we were able to determine any existing links in the

literature between these genes. ANGPT1, HIF1A and NOS2 were shown to be linked via the 'HIF-1 signalling pathway', which is the predominant signalling pathway in the cell's regulation of hypoxia; this was supported in the literature by a single publication (Lavine *et al.* 2013). Multiple publications were present linking paired combinations of these genes (ANGPT1 & HIF1A, HIF1A & NOS2 or ANGPT1 & NOS2) but only one paper listed all three genes in combination. FGF2, HIF1A and NOS2 were linked via 'Pathways in cancer' and this was supported in the literature (Dinarello *et al.* 2011, Lavine *et al.* 2013, Lehner *et al.* 2011) although as with the previous gene combination, there were more publications about pairs of these genes than the full combination. FGF2 and ANGPT1 were shown to be linked by the 'Rap1 signalling pathway', the 'Ras signalling pathway' and the 'PI3K-Akt signalling pathway' with numerous publications supporting their linkage (De Leon *et al.* 2014, Neve *et al.* 2014, Stockmann *et al.* 2014, Talwar and Srivastava 2014). The 'Rap1 signalling pathway' is controlled by Rap1, an enzyme of the GTPase family which is responsible for the control of cell-cell and cell-matrix interactions and has a specific role in cell adhesion, cell-cell junction formation and cell polarity. The 'Ras signalling pathway' is controlled by small GTPase enzymes which are predominantly involved in intracellular signal transduction. This pathway controls cellular outcomes such as cytoskeleton organisation, cell motility and migration or cell growth, cell survival and cell death. The 'PI3K-Akt signalling pathways' have been shown to link a number of fundamental cellular functions such as protein synthesis, cell survival, cell growth, cell proliferation, angiogenesis, cell metabolism and cell cycle progression.

FGF2 and HIF1A were linked by the 'Proteoglycans in cancer' pathway and a number of publications (Chung *et al.* 2012, De Leon *et al.* 2014, Jiang *et al.* 2014, Riabov *et al.* 2014, Stockmann *et al.* 2014, Talwar and Srivastava 2014, Wise *et al.* 2012), whilst HIF1A was linked to NOS2 via a curate pathway published in NCI-Nature Pathways

Interaction Database (<http://www.ndexbio.org/>), the ‘HIF-1 signalling pathway’, ‘Pathways in cancer’ and in the literature by publication (Bao *et al.* 2012, Elks *et al.* 2013, Lehner *et al.* 2011). ANGPT1, FGF2 and HIF1A were linked in numerous publications (De Leon *et al.* 2014, Hadjipanayi and Schilling 2013, Peplow and Baxter 2012, Riabov *et al.* 2014, Stockmann *et al.* 2014, Talwar and Srivastava 2014) but were not linked by a known signalling pathway. There were no signalling pathways linking all four genes together and only a single publication in the literature (Lavine *et al.* 2013) that has examined all four of these genes together in one investigation and found a link between them. The link between these genes is angiogenesis induced by ischemia or lack of oxygen (which in a general tissue would be referred to as hypoxia). Although we are unable to determine whether there was an up- or down-regulation of expression in these genes, which would enable us to compare trends in gene expression with those outlined by Lavine *et al.* (2013) directly; we can hypothesise at this juncture that the application of an orthodontic force for any duration of time may result in hypoxia and induce an angiogenic response.

STRING and KEGG were used to identify any pathways with which the borderline significant genes were associated. ITGAV, KDR and PDGFA were shown to be associated with the ‘PI3K-Akt signalling pathway’ and the ‘Focal adhesion’ pathway. KDR and PDGFA were shown to be associated with the ‘Rap1 signalling pathway’, the ‘Ras signalling pathway’ and the ‘Cytokine-cytokine receptor interaction’ pathway. ITGAV and PDGFA were shown to be linked by the ‘Regulation of actin cytoskeleton’ pathway whilst ITGAV and KDR were linked by the ‘Proteoglycans in cancer’ pathway and the curate pathway ‘VEGFR2 (dimer)/ VEGFA (dimer)/ ITGAV-ITGB3/ Vitronectin’ published on the NCI-Nature Pathways Interaction Database (<http://www.ndexbio.org/>). ITGAV and TGFBR1 were linked via curate pathways ‘Complex assembly’ and ‘Complex: ITGAV-ITGB8/TGFBR1’ published on the NCI-

Nature Pathways Interaction Database (<http://www.ndexbio.org/>). As evidenced from the data available from current literature, a number of the genes with borderline significance were linked to those with statistical significance through a variety of signalling pathways. The pathways that these genes are linked together by suggest that there is an alteration within the tissue directly associated with cellular adhesion, intracellular signalling, cell survival and angiogenesis in response to orthodontic force application. It is hypothesised that these genes would also be involved in the angiogenic response of the human dental pulp to hypoxia induced by orthodontic force application but may require a longer duration than two weeks of force application or perhaps a greater intensity of force in order to obtain a level of statistical significance.

When comparing force application for 1 day with force application for 3 days, there was shown to be a statistically significant difference in the expression of ANGPT1 and HIF1A; and a borderline significant difference in the expression of FGF2, ITGAV and NOS2. Although the analysis does not indicate whether these genes are up- or down-regulated in response to a longer duration of force, these findings are important as they provide an indication as to possible tissue responses of the pulp in reaction to routine orthodontic treatment. ANGPT1 and HIF1A are not linked by any known signalling pathways but are linked in the literature to angiogenesis (De Leon *et al.* 2014, Forget *et al.* 2014, Riabov *et al.* 2014, Stockmann *et al.* 2014) and hypoxia (Fan *et al.* 2014, Hadjipanayi and Schilling 2013). FGF2, ITGAV and NOS2 are all linked by the 'Pathways in cancer' signalling pathway and in the literature to angiogenesis in HIV-infected patients (Rasheed *et al.* 2009).

Between the next duration of force, 3 days to 2 weeks, there was shown to be a significant difference in the mRNA expression of the genes ANGPT1, FGF2, HIF1A, ITGAV, KDR, PDGFA, TEK and TGFBR1. There was also a borderline significant difference in the mRNA expression of ITGB3 and MMP2. There was no signalling

pathway or literature that linked all 8 of the significantly differentially expressed genes between these two experimental time-points; however, a number of genes were linked by specific signalling cascades and in the literature. ANGPT1, FGF2, ITGAV, KDR, PDGFA and TEK were all linked by the 'PI3K-Akt pathway' but a maximum of five of these genes were shown to be linked in a single publication at any one time. The genes were linked in the literature to hypoxia, vascular differentiation and angiogenesis (Burns *et al.* 2011, Carmeliet and Collen 1998, Deng *et al.* 2006a, Deng *et al.* 2006b, Paternostro *et al.* 2010, Rahbari *et al.* 2011, Tressel *et al.* 2007, Vacca *et al.* 2003). ANGPT1, FGF2, KDR, PDGFA and TEK were linked by the 'Rap1 signalling pathway' and the 'Ras signalling pathway' in addition to the 'PI3K-Akt signalling pathway' and were linked in the literature to angiogenesis (Rahbari *et al.* 2011, Tressel *et al.* 2007).

ITGB3 and MMP2 although shown to be effected in a borderline significant manner as a response to orthodontic force application, are not linked to each other in the literature or by any known pathway. They were however linked to known signalling cascades and published works when considered with the other significantly differentially expressed genes at this time-point of force application. ITGB3 was shown to be linked to ITGAV and FGF2 in the 'Syndecan interactions' pathway and to ITGAV and KDR in the curated pathway 'VEGFR2 (dimer)/ VEGFA (dimer)/ ITGAV-ITGB3/ Vitronectin' published on the NCI-Nature Pathways Interaction Database (<http://www.ndexbio.org/>). ITGB3, linked with these other genes in the literature was shown to be directly related to angiogenesis (Bhuvaneswari *et al.* 2008, Carmeliet and Collen 1998, Hanjaya-Putra *et al.* 2011, Hayashi *et al.* 2008, Mottet *et al.* 2007, Niland and Eble 2012, Rivera and Brekken 2011, Yao *et al.* 2006). MMP2 was shown to be linked to the significantly different genes by the 'Pathways in cancer' and the 'Proteoglycans in cancer' signalling pathways. It has been shown in the literature to be associated with hypoxia and

angiogenesis (Deng *et al.* 2006a, Deng *et al.* 2006b, Glesne *et al.* 2006, Lewis and Pollard 2006, Paternostro *et al.* 2010, Vacca *et al.* 2003).

Over the entire experiment, from 1 day of force application to 2 weeks of treatment, there was shown to be a statistically significant difference in the mRNA expression of NOS2. HIF1A, KDR and TGFBR1 mRNA expression were also affected by the application of orthodontic force for the full period of 2 weeks, although this was less significant statistically when compared to the changes in NOS2 mRNA expression.

NOS2 is an inflammatory mediator and is known to be associated with several of the signalling pathways previously mentioned, i.e. 'Proteoglycans in cancer' and the 'Rap1 signalling pathway'. It has shown to be prevalent in the literature amongst studies of cellular stress (Verma *et al.* 2014, Zhang *et al.* 2014), hypoxia (Branco-Price *et al.* 2013, Cowburn *et al.* 2013, Lehner *et al.* 2011), cell death (Iwashina *et al.* 1998) and angiogenesis (Breton-Romero and Lamas 2014, Singh *et al.* 1996). HIF1A, KDR and TGFBR1 were shown to be related in the literature but not by any known signalling pathways. These genes were shown to be associated with hypoxia (Ciucan *et al.* 2011, Porter *et al.* 2014), angiogenesis (Albini *et al.* 2007, Ciucan *et al.* 2011, Ozkan *et al.* 2012, Porter *et al.* 2014, Tressel *et al.* 2007) and erythrocyte differentiation (Fraser 2013).

It is important to note at this point in time that whilst a number of genes have been shown to be responsive to various durations of force application, the links in the literature and signalling pathways are for protein-protein interactions and not for mRNA. We have hypothesised prior to investigation that an application of orthodontic force will induce an angiogenic response as previously demonstrated by Derringer *et al.* (Derringer *et al.* 1996, Derringer and Linden 1998, Derringer and Linden 2003, Derringer and Linden 2004, Derringer and Linden 2007) but it is important to consider that prior to the translation of proteins from up-regulated mRNA, multiple post-

transcriptional regulatory processes occur which may eliminate or enhance the translation of a given protein. We maintain that the genes shown to be effected by an application of orthodontic force are linked to an angiogenic response within the pulp but cannot conclude at this juncture that an application of orthodontic force will result in increased angiogenic protein.

4.5.7 Is the mRNA Expression of Angiogenesis-Specific Genes in the Human Dental Pulp Linked?

A factor analysis was performed by Dr Nora Donaldson in order to determine whether any commonality in gene expression variability existed across all time-points of treatment. This was done despite there being an undeterminable baseline of expression for the GOIs due to the large amount of biological variability present. Significance was set at 5 % and analysis was performed on normalised C_q data. By performing this analysis we were able to determine that mRNA expression for the genes MMP2, TGFB1 and VEGFA was the most tightly correlated and that expression would change in the same direction, i.e. if MMP2 was up-regulated, TGFB1 and VEGFA would also be up-regulated. By STRING analysis (www.string-db.org) MMP2, TGFB1 and VEGFA were shown to be linked via the 'Pathways in cancer' and 'Proteoglycans in cancer' signalling pathways. They were also shown to be linked in the literature to angiogenesis (Riabov *et al.* 2014), inflammation (Landskron *et al.* 2014) and hypoxia (Angelini *et al.* 2013). As VEGFA is a mitogenic factor specific to endothelial cells; MMP2's primary function is the degradation of proteins in the extracellular matrix to regulate neovascularization and TGFB1 is an anti-inflammatory cytokine; the finding that these genes are tightly correlated in response to orthodontic force application over a duration of 1 to 14 days suggests that the human dental pulp may undergo an amount of oxidative stress, inducing inflammation and an angiogenic response to regulate for these

changes; or perhaps an inflammatory response to orthodontic treatment leads to hypoxia in the pulp followed by a subsequent regulatory angiogenic response.

ANGPT1, FGF2, HIF1A, ITGAV, ITGB3, KDR, PDGFA, PDGFB, ROBO4, SELE, TEK and TGFBR1 were all shown to be correlated and to change in the same direction. According to STRING analysis (www.string-db.org), where gene co-expression was assessed, there was no co-expression of these genes found amongst Homo sapiens. The only co-expression found was in the mouse model between TEK and ROBO4. Despite this finding, a number of these genes are known to be linked within the literature (Carmeliet and Collen 1998, Fan *et al.* 2014, Murakami 2012, Paternostro *et al.* 2010, Riabov *et al.* 2014, Stockmann *et al.* 2014, Talwar and Srivastava 2014).

We have found evidence in the literature to contest the finding that ANGPT1, PDGFB and TEK are correlated and will be up- or down-regulated concurrently; all other genes were shown to increase or decrease in expression while linked to the similar response of the remaining genes in the panel, according to the literature. TEK and PDGFB are known to be linked through the Rap1, Ras and PI3K-Akt signalling pathways (www.string-db.org) and a down-regulation of PDGFB has previously been associated with a collaborative regulation by ANGPT1 and TGFBR1 (Nishishita *et al.* 2004). PDGFB has also been shown to be down-regulated by increased TEK expression in Human Umbilical Vein Endothelial Cells (HUVEC) (Uebelhoer *et al.* 2013). Therefore as ANGPT1 increases, TEK is thought to increase and thus results in a decrease in PDGFB expression.

It is interesting to note that should HIF1A be up-regulated in response to hypoxia, induced by an application of orthodontic force, the remaining genes which are known to be associated with inflammation and angiogenesis will also be up-regulated in a correlated fashion.

IL8 and NOS2 were the only genes found not to be correlated to any of the other GOIs under our experimental conditions and were independent of each other, i.e. as IL8 was up-regulated; there was neither up-regulation nor down-regulation of NOS2 mRNA. This was not expected as NOS2 has previously been shown to enhance the synthesis of IL8 (Vuolteenaho *et al.* 2009) and gene expression should not only have been linked but should have been expressed in a similar direction, according to this publication. IL8 is also a pro-inflammatory chemokine and there was an expected association with TGFB1 as not only did TGFB1 show the potential to link inflammation to angiogenesis and hypoxia in the orthodontically treated pulps but IL8 has previously been shown to be regulated by TGFB1 in human prostate cancer cells (Lu and Dong 2006). The lack of correlation between these genes and those of the rest of the panel are not understood at this time and further investigation is required in order to fully understand the changes in mRNA expression in the human dental pulp during routine orthodontic treatment.

4.5.8 Correcting for Biological Variability Allows for Statistically Valid Conclusions to be Drawn

Following multiple, varied analyses of the normalised C_q data in order to determine the effects of orthodontic force duration or patient phenotypic factors on the mRNA expression of the GOIs, we came across a methodology in the literature which would eliminate the effects of biological variability on the mRNA expression data. The methodology outlined by Willems *et al.* (2008) had previously been used in microarray data transformation to identify a “robust and powerful method to standardise independent biological replicate experiments so as to draw out statistically sound conclusions from a dataset that otherwise was of limited value due to high inter-experimental variation.”. A copy of the published literature (Willems *et al.* 2008) and our spreadsheet of formulae defining our understanding of the methodology was sent to

Dr Nora Donaldson for clarification prior to analysing raw C_q values as a number of steps within the method had not been included in the publication of Willem *et al.*'s (2008) paper. We have outlined the full methodology used in **Chapter 8, section 8.2.1** Following analysis with the variability correction method, we were able to observe statistically significant changes in mRNA expression at every time-point of orthodontic force application. These findings were in agreement with those outlined by the original authors (Willems *et al.* 2008) where initial analysis by Livak *et al.*'s (2001) method yielded no significant results but additional standardisation cancelled out high levels of inter-experimental variation and enabled the observation of statistically significant differences in the various treatment groups.

By comparing the graphs for the REST analyses (**Figure 14 – Figure 16**) against those created following the correction for biological variability (**Figure 17 – Figure 19**) it was clear to see that the trends observed in the REST analyses were equivalent to those present in the variability-corrected samples. These findings further substantiated the relevance and accuracy of Willem *et al.*'s (2008) method and Dr Nora Donaldson's clarification of their processes in order to obtain statistical significance.

There was shown to be a statistically significant up-regulation of TEK mRNA and significant down-regulation of PDGFB mRNA following a period of 1 day's orthodontic treatment (**Figure 17**). TEK and PDGFB are known to be linked through the Rap1, Ras and PI3K-Akt signalling pathways (www.string-db.org) and a down-regulation of PDGFB has previously been associated with a collaborative regulation by ANGPT1 and TGFB1 (Nishishita and Lin 2004). PDGFB has also been shown to be down-regulated by increased TEK expression in HUVEC (Uebelhoer *et al.* 2013). It is thought that stimulation of TEK by ANGPT1 results in the downstream phosphorylation of Akt and that this process is necessary for the maintenance of the vasculature as PDGFB is a vascular smooth muscle cell (vSMC) attractant and would result in

numerous attachments of pericytes on the vessel outer walls, should this process not be regulated. In order to validate our findings according to the publication by Nishishita and Lin (2004), we would have to see an increase in expression of both ANGPT1 and TGFB1. This was not the case, as there was only an observed increase in ANGPT1 expression. Therefore, we can justify our findings according to the publication by Uebelhoer *et al.* (2013) where TEK is stimulated by ANGPT1 resulting in downstream down-regulation of PDGFB. We had originally hypothesised that there would be a greater change in expression amongst a wide panel of genes following the application of an orthodontic force for 24 hr but here we see an almost homeostatic process being induced as a response to treatment. Cytokines and chemokines associated with the inflammatory response were all down-regulated at this time-point of force application and HIF1A expression was shown to marginally increase. Although the expression of HIF1A was not statistically significant, this trend may indicate the inception of hypoxia in the human dental pulp as an immediate response to orthodontic force application.

After 3 days of orthodontic treatment, pulps exhibited a general trend of down-regulation in mRNA expression, with a statistically significant down-regulation of ANGPT1, HIF1A, KDR, PDGFA, PDGFB, ROBO4, TEK and TGFBR1 mRNA (**Figure 18**). This result corroborates our previous findings from the factor analysis (**Table 64**), which showed that these genes are loosely correlated and will increase or decrease in a like manner.

A down-regulation of ANGPT1 expression has been linked in the literature to hypoxia, and therefore to an increase in HIF1A expression (Tsuzuki *et al.* 2013). Our findings are contrary to this in that we have observed a decrease in both ANGPT1 and HIF1A in response to 3 days of routine orthodontic treatment. In another study, HIF1A was shown to be linked to the activation of angiogenic growth factors including ANGPT1, PDGFB and VEGFA (Manalo 2005), indicating that these genes are correlated and will

increase/decrease in a linked manner as per our findings in the factor analysis. Not enough literature is available to make a conclusive statement as to either corroborate or invalidate our findings with respect to the behaviour of these two genes at this treatment time-point.

It has been shown in the literature that an increase in phosphorylated KDR will occur simultaneously with an increase in HIF1A (Giatromanolaki *et al.* 2008), therefore our finding that these two genes are down-regulated together suggests that the angiogenic and hypoxic responses may be linked in the human dental pulp's reaction to orthodontic treatment.

PDGFA and PDGFB have been shown to be expressed at different rates but also have been shown to be expressed at the same rate in a similar direction; depending on the cell type (Barrett and Benditt 1988). The correlation seen amongst the panel of genes that have been investigated in our study may relate not only to function of the gene within the cell but also to cellular composition.

PDGFB and ROBO4 were shown to be concurrently down-regulated in the literature in a state of hyperoxia (Ishikawa *et al.* 2010). Hyperoxia is an excess of oxygen or increased partial pressure of oxygen in a tissue and is an opposing condition to hypoxia. Should hyperoxia exist in the human dental pulp at 3 days of force application, we should expect a down-regulation of HIF1A, KDR and TGFBR1 mRNA expression according to the literature (Lin *et al.* 2005, Moen *et al.* 2009, Ozaki *et al.* 1999). Our findings corroborate the expected down-regulation of mRNA expression for these genes and suggest that a state of hyperoxia may exist in the human dental pulp at 3 days of orthodontic force application.

It was surprising to note that following 3 days of treatment PDGFB and TEK were concurrently down-regulated. We have previously shown evidence from the literature to substantiate our findings at 1 day of force application that as TEK increases, PDGFB is

down-regulated (Uebelhoer *et al.* 2013). As ANGPT1 and TEK have been strongly linked in the literature (Augustin *et al.* 2009, Kuroda *et al.* 2001, Thomas and Augustin 2009, Wehrle *et al.* 2009) and have been linked in both the factor analysis and the variability corrected relative expression analysis, we hypothesise that the mechanism by which PDGFB is regulated at 3 days of force application must be different to the homeostatic mechanism outlined by Uebelhoer *et al.* (2013) which supports our findings at 1 day of force application.

There was an observed increase in the expression of IL8 at this stage of routine orthodontic treatment and although this increase in mRNA expression did not reach statistical significance, it is imperative to note that IL8 has only been isolated from dental pulps in a state of inflammation. IL8 has also been shown to be linked to hyperoxia (Hjort *et al.* 2003, Lee and Kim 2011) and we hypothesise that following the inception of orthodontic force application, there is a state of hypoxia which induces an inflammatory and angiogenic response in the pulp. Due to the morphology of the pulp, with closed apices, there may be an over compensatory affect of the angiogenic or inflammatory response which results in hyperoxia within the tightly encapsulated tissue. This hypothesis requires verification by light microscopy to investigate whether there is an observable increase in the vasculature of the treated pulps between 1 and 3 days of force application that could support our proposed reaction of the pulp to treatment.

After 2 weeks of orthodontic treatment there was a general trend towards up-regulation of mRNA expression in the GOIs (**Figure 19**). There was shown to be a statistically significant difference in HIF1A, ITGAV, KDR and TGFBR1 mRNA expression. When analysed using STRING (www.string-db.org), only three of the four genes were shown to be linked by known signalling pathways whilst pairs of genes were shown to be associated via other known pathways. ITGAV, KDR and HIF1A were shown to be linked via the 'Proteoglycans in cancer' signalling pathway while ITGAV was paired

with HIF1A via both the 'Thyroid hormone signalling pathway' and 'Pathways in cancer'. ITGAV was linked with KDR through the 'Focal adhesion' pathway, the 'PI3K-Akt signalling pathway' and the curated pathways for 'Complex: VEGFR2(dimer) /VEGFA(dimer)/ Hsp90/ ITGAV/ ITGB3/ Victronectin' and 'Complex: ITGAV/ ITGB3/ VEGFR2/ VEGFA(dimer)' published on the NCI-Nature Pathways Interaction Database (<http://www.ndexbio.org/>). ITGAV was paired with TGFBR1 through the curated pathways: 'Complex Assembly' and 'Complex: ITGAV/ITGB8/TGFBR1' published on the NCI-Nature Pathways Interaction Database (<http://www.ndexbio.org/>). There was no signalling pathway evidence to suggest that KDR or HIF1A were linked with TGFBR1.

ITGAV, HIF1A and KDR were all shown in the literature to be up-regulated together in endometrial samples from patients with ovarian endometriosis, where eutropic endometrial tissue is hypothesised to have an abnormal angiogenic potential (Laudanski *et al.* 2014). Whilst HIF1B is constitutively expressed in the cell, HIF1A is only up-regulated in response to hypoxia (Wang *et al.* 1995), suggesting that following two weeks of force application, the human dental pulp is in a state of oxidative stress and that an angiogenesis is being initiated as a response to this stress state.

ITGAV and KDR increases were linked in the literature to angiogenesis (Avraamides *et al.* 2008) and to coagulation (D'Asti *et al.* 2014). As it has previously been observed that orthodontic force application has resulted in congested vasculature of the human dental pulp (Lazzaretti *et al.* 2014, Mostafa *et al.* 1991), this increase in mRNA expression associated with coagulation may confirm previous findings but has yet to be investigated at the proteomic level.

At this time-point of treatment, there was also an increase in the mRNA expression of IL8 and NOS2; although neither of these reached a level of statistical significance it is important to note that these genes are only increased in cases of inflammation and we

hypothesise that in addition to a state of oxidative stress, the human dental pulp is in an inflammatory state at two weeks of routine orthodontic treatment.

It is interesting to note that at two weeks of orthodontic force application, there was a decrease in the expression of VEGFA and TGFB1 mRNAs. These two genes are normally at the forefront of the angiogenic response and it may be the case that while hypoxia is present, the angiogenic response has only begun to be initiated. Further work will need to be carried out in a strict time-course from 14 days of treatment onward to be able to ascertain true response mechanisms in the pulp.

4.5.9 Determining Changes in Protein Expression by ELISA

Where possible, protein analysis was performed on samples obtained from patients previously assigned to the mRNA expression analyses in order to reduce the introduction of further biological variability to the sample data. It has been shown that maxillary and mandibular teeth respond in a similar manner to orthodontic force application, with respect to cellular respiration (Hamersky *et al.* 1980) and it was therefore assumed that protein and mRNA expression results within patients would be relatively comparable.

A custom multiplex ELISA (Quantibody® Array) was created and performed by RayBiotech Inc., Georgia, USA for 10 of the 17 genes analysed at the transcriptomic level. These genes were selected based upon availability and relevance to the original protein studies performed by Derringer *et al.* (Derringer and Linden 2003, Derringer and Linden 2004). Due to the size of the assay plate, only 5 patients were selected for each treatment time-point.

It was shown in all samples, across all treatment time-points that FGF2 was the only protein expressed in pulp samples above the lower LOD for the ELISA assay. This was not expected as despite our previous observations, where human dental pulps yield low

concentrations of mRNA (**Chapter 3**), we had supposed that due to the cellular nature of the tissue, protein concentrations would be greater and in particular, more abundant than the low concentrations at which the ELISA assay limits were set. It was confirmed by RayBiotech Inc., Georgia, USA that the 1 ml sample aliquots had been delivered to them on dry ice and had not thawed or denatured during transportation. The original BCA assay concentrations were verified and were compared to guidelines for assay sensitivity where the assay was shown to have a detection limit between 20 – 2000 µg/ml (Johnson 2012). According to the BCA assay results, total protein concentrations extracted from the pulp samples were in the range of 71799.31 – 527162.63 µg/ml. This was well above the BCA assay's LOD and in contradiction to the low concentrations observed in the ELISA, which was performed on sample aliquots with a standardised concentration of 2 µg/µl. It should be stressed at this point that the BCA assay does not only measure the concentration of a limited selection of genes but rather, measures the concentration of total extracted protein from the tissue homogenates. Concentrations of the GOIs were found to be extremely low in the tissue homogenate by the ELISA and it is proposed that in order to adequately quantify changes between control and orthodontically treated pulp samples, so as to make conclusive statements regarding the tissue's response to treatment, a high-sensitivity assay such as fluorescent amplification catalysed by T7 polymerase technique (FACTT) should be utilised instead (Zhang *et al.* 2006).

A paired Student's t-Test was performed on the FGF2 results and there was shown to be no statistically significant difference in the protein expression of this gene between the treated and the control groups at any of the experimental time-points of orthodontic treatment. Unlike high-sensitivity microarray or mass spectrometry experiments where normalisation for inter-sample and inter-experimental variation is considered (Kreil *et al.* 2004, Ting *et al.* 2009), no such correction is currently available for ELISA data.

Analyte data was normalised against the total input of analyte per reaction ($\mu\text{g/ml}$) and concentrations of analyte present in the sample (pg/ml) were calculated from a standard curve of positive control for the protein of interest. As no baseline of cellular activity in the control samples could be determined, each patient's contralateral pair should be compared qualitatively and individually, so as not to make conclusive remarks with respect to the increased or decreased expression of proteins as a response to force application.

When sample pairs that expressed analyte concentrations greater than the assay's lower LOD were assessed qualitatively, it was observed that FGF2 abundance increased at 1 day and 2 weeks of treatment but decreased at 3 days. This trend matches that which was observed in the mRNA expression data following the correction for biological variability. This suggests that an increase in FGF2 mRNA expression yields a direct increase in FGF2 protein abundance (Moffett *et al.* 1998) and that FGF2 is translated to protein in the human dental pulps with minimal post-transcriptional modification as there appears to be little time lapse between transcription and translation.

FGF2 is a pleiotropic factor and acts in a paracrine, intracrine and autocrine manner (Delrieu 2000, Gomm *et al.* 1997, Kim and Ma 2013, Lefevre *et al.* 2009, Presta *et al.* 2009, Stachowiak *et al.* 1997). It has been shown to be associated with inflammation, angiogenesis, neuronal growth, neurotransmitter synthesis and the response to neuropathic pain (Mocchetti *et al.* 1996, Presta *et al.* 2005, Presta *et al.* 2009, Yamanaka *et al.* 2007). With respect to angiogenesis and inflammation, FGF2 is known to regulate the expression of extracellular matrix components, induce neovascularisation, stabilise vasculature and recruit pericytes through the induced expression of PDGFB and TGFB1 (Andres *et al.* 2009, Presta *et al.* 2005, Presta *et al.* 2009). We have previously suggested in our mRNA expression analyses that orthodontic force application may induce hypoxia, angiogenesis and inflammation

within the human dental pulp; although this has yet to be confirmed and the order in which these three responses occur are yet to be determined. The observed increase in FGF2 protein abundance in treated human dental pulps at the 1 and 14 day time-points may link to our hypotheses as it has been shown in the literature that “inflammatory mediators can activate the endothelium in order to synthesise and release FGF2 which will stimulate angiogenesis by an autocrine mechanism” and may lead to an increase in interstitial fluid and hypoxia (Kuwabara *et al.* 1995, Presta *et al.* 2009, Wang *et al.* 2000). As the results from the ELISA are only able to be assessed qualitatively due to the lack of variability correction protocols and a number of samples falling below the LOD, no direct correlation can be drawn between our observations and our hypothesis. Yet, should inflammation occur and angiogenesis be induced, we would expect to see an increase in PDGFB and TGFB1 proteins which would correlate with pericyte recruitment and increased interstitial fluid pressure. We can make no comment as to the pulp’s response with respect to hypoxia as HIF1A was not available for analysis on the ELISA plate. We do however confirm the findings of Derringer *et al.* (Derringer and Linden 2003, Derringer and Linden 2004), where an application of orthodontic force for a period of 2 weeks will result in increased FGF2 protein abundance in the human dental pulp; and the findings of Tran-Hung *et al.* (2006) who have identified that FGF2 increases in the human dental pulp following injury.

With respect to the other angiogenic growth factors assessed by Derringer and Linden (2003, 2004), there were no distinguishable trends that we could observe from the TGFB1 or VEGFA ELISA data due to concentrations in the tissue homogenates being below the assay’s lower LOD and the majority of the sample pairs having a negative result from either one or both of the pulps. Our findings do not discredit those of Derringer and Linden (2003, 2004) however, as this group utilised neutralising antibodies to confirm the presence of secreted growth factors. Any amount of growth

factor present would have been neutralised by these antibodies to some extent and as we were unable to determine the concentrations with an ELISA, we cannot state conclusively that there is no increase in TGF β 1 or VEGFA production or secretion but rather that the analytes present are well below our assay's limits of detection. We propose that further work be carried out with high-sensitivity assays in order to determine concentrations and relative changes in abundance of these growth factors before a conclusive statement is made.

There was no observable change in protein abundance of PDGFB following 1 day of force application between control and treated samples. Protein abundances increased at 3 days of treatment and then decreased by 2 weeks of treatment. These findings are in contrast to our mRNA expression analyses which showed a decrease in PDGFB mRNA at treatment days 1 and 3 and no distinguishable change between the control and treated samples at 2 weeks of treatment. PDGFA protein abundance was shown to decrease at all three time-points of force application which also contradicts the mRNA expression findings. In the variability corrected mRNA expression analysis, it was seen that PDGFA is down-regulated at 1 and 3 days before being up-regulated at 2 weeks of force application.

PDGF is a dimeric glycoprotein that can be expressed as either of the homodimers PDGF-AA or PDGF-BB; or as the heterodimeric PDGF-AB (Kaetzel 2003, Meyer-Ingold and Eichner 1995, Shih and Holland 2006). All isoforms are known to bind to the receptor PDGFRB, while receptor PDGFRA binds only the PDGF-AA homodimer (Heldin *et al.* 1989, Heldin and Westermark 1999, Kaetzel 2003, Shih and Holland 2006). In our ELISA analysis, we attempted to quantify the concentrations of the homodimers, PDGF-AA and PDGF-BB, while PDGF-AB was not considered due to unavailability from the supplier.

It is well established that PDGFB plays a significant role in angiogenesis, particularly in stabilisation of the neovasculature by pericyte recruitment (Abramsson *et al.* 2003, Andrae *et al.* 2008, Gianni-Barrera *et al.* 2014, Lindblom *et al.* 2003, Wang *et al.* 2012). It has also been shown to play a role in fibrotic diseases, such as atherosclerosis (Libby *et al.* 1988), pulmonary fibrosis (Abdollahi *et al.* 2005) and liver cirrhosis (Zhou *et al.* 2014), which are resultant of inflammation in the tissues. In addition to its role in the inflammatory response, it has been documented that PDGFB plays a crucial role in the homeostasis of interstitial fluid pressure (Heuchel *et al.* 1999, Wiig *et al.* 2003) and is responsible for increasing pressure to a point that may result in interstitial hypertension (Pietras *et al.* 2001, Reed and Rubin 2010).

We observed an abundance of this protein following 3 days of force application, a time-point where we have previously hypothesised that the tissue is in a state of hyperoxia. According to the literature, PDGFB mRNA and proteins are suppressed by hyperoxia (Zhang *et al.* 2005). This suggests that hyperoxia appears to be a less likely cause of the transcriptomic and proteomic differences observed in treated pulps at this time-point of treatment. Should hyperoxia not occur at this experimental time-point, the additional differences observed in the mRNA and protein abundances indicate that pulps undergoing routine orthodontic treatment are in an inflammatory state at 3 days of treatment. The increased PDGFB abundance would compliment this hypothesis of an inflamed pulp and may substantiate our finding of increased HIF1A mRNA expression in the two week samples; should an increase in PDGFB protein at 3 days of treatment result in increased interstitial fluid pressure in the tightly encapsulated tissue and lead to oxidative stress and a possible hypoxic response. We have already identified an increase in FGF2 analyte abundance following 1 day of force application and have noted that increased FGF2 is responsible for the regulation of PDGFB, resulting in increased

interstitial fluid pressure (Presta *et al.* 2009). This compliments our finding of increased PDGFB analyte abundances at 3 days of force application.

We propose that further investigation be carried out in order to determine the changes in interstitial fluid pressure, the incidence of inflammation and the possible hypoxic response to this inflammation in a time-course of force application in order to make any conclusive statement as to the pulp's response to orthodontic treatment. We also propose that a larger group of patients be investigated in order to determine true changes; as five patients per treatment group, where known large-scale biological variability exists, is only representative of a preliminary screening of the true physiological response.

The PDGFA and PDGFB protein chains are subject to different regulatory mechanisms and are produced by different cell types in differing ratios (Dirks and Bloemers 1995, Hoch *et al.* 2003, Kaetzel 2003, Meyer-Ingold and Eichner 1995, Shih and Holland 2006). This has been demonstrated further by Rahmann *et al.* when investigating osteoclastogenesis, where all three isoforms of PDGF were analysed and found to be secreted at distinct stages of bone generation (Rahman *et al.* 2015). PDGFA is required for epithelial cell proliferation and has been associated with increased proliferation of mesenchymal stem cells but is primarily active during embryogenesis (Landskron *et al.* 2014, Mizuno *et al.* 2015, Sukhotnik *et al.* 2012). At all three experimental time-points, we observed a decrease in PDGFA analyte expression in treated samples compared with untreated controls. There is no evidence in the literature to suggest that inflammation, hyperoxia or hypoxia would result in decreased protein expression for this GOI. We therefore propose that in order to make a conclusive statement as to the effect of orthodontic force application on PDGFA protein expression, a larger group of patients needs to be examined. The differences between control and treated pulps in the protein abundance analysis appear to contradict the results obtained from the variability

corrected mRNA analysis, where there was an increase in mRNA expression following two weeks of force application.

We have previously suggested that an increase in PDGFB protein, as a potential result of orthodontically induced inflammation, may alter the interstitial pressure of the tissue and induce hypoxia at the two week time-point. It has also been documented that an increase in PDGFB mRNA will precede an increase in PDGFA mRNA in hypoxic vascular remodelling (Katayose *et al.* 1993) and in wound healing (Pierce *et al.* 1995). This suggests that despite our observing a decrease in PDGFA analyte concentration at two weeks, the increase in PDGFA mRNA expression is linked to the increased PDGFB expression within our time-course; and that the changes observed in the pulpal response to force application correspond with published reactionary mechanisms of inflammation resulting in increased interstitial fluid pressure and possible hypoxia.

As no conclusive statement can be made from our qualitative data analysis in order to corroborate or repudiate the findings of Derringer and Linden (2003, 2004), we propose that the activity of both receptors, PDGFRA and PDGFRB, be assessed; as Derringer and Linden's studies utilised blocking antibodies for PDGF and did not specify whether PDGFA or PDGFB was being secreted by the pulps in response to routine orthodontic treatment.

Our study also showed an increase in the protein abundance of SELE at all time-points of treatment; this was in contradiction to the changes observed in the variability corrected mRNA expression data where at 1 and 14 days there was no perceivable change relative to the control samples; and in the 3 day treated samples where mRNA expression was down-regulated in response to force application. SELE protein is synthesised when vascular endothelial cells are stimulated by pro-inflammatory cytokines (Kawase *et al.* 2009), in order to attract, bind and activate circulating leukocytes to the site of inflammation or injury (Kraiss *et al.* 2003, Winkler *et al.* 2012).

The increase in the expression of this analyte observed in the treated samples, relative to untreated controls suggests that orthodontic force application induces inflammation within the human dental pulp. In the mRNA expression analysis, we noted an increase in pro-inflammatory chemokine IL8 at days 3 and 14 of treatment, with an additional increase in NOS2 expression at 14 days of force application. These mRNA expression changes, confirm that inflammation may be occurring in the dental pulp as a response to routine orthodontic treatment. However, no clear conclusion can be made at this time as protein concentrations for these GOIs were not investigated due to the unavailability of these genes in the ELISA reaction.

4.5.10 Investigating Changes within Individuals to Determine the Effect of Force Application on Protein Expression at Distinct Treatment Time-Points

Further qualitative assessment was performed in collaboration with Dr Nora Donaldson in order to determine whether trends in expression at any of the time-points could be observed. Sample pairs where one of the two samples gave an undeterminable reading of 0 pg/ml were excluded but all other samples whether above or below the assay's lower LOD were considered.

Following 1 day of orthodontic treatment there was a weak trend towards increased FGF2, TEK and SELE protein abundance. This general trend was loosely correlated with the trend observed in the variability corrected mRNA expression data. While no change was observed for SELE mRNA and there was only a minor increase in the mRNA expression of FGF2, TEK mRNA showed a significant up-regulation at this time-point of treatment. We have previously discussed that an increase in TEK and ANGPT1 mRNA expression is associated with the homeostatic regulation of PDGFB for pericyte recruitment (Nishishita and Lin 2004, Uebelhoer *et al.* 2013). When re-

examining the protein data, there was an observable but weak trend in increased ANGPT1 protein expression but no determinable trend in PDGFB expression (owing to negative results from the ELISA). As a result of the absence of observable protein trends for PDGFB, we cannot relate the changes in TEK protein abundance to the homeostatic mechanisms discussed in the literature. FGF2 and SELE have already been discussed previously in the context of inflammation and we have hypothesised that in a response to orthodontic force application, the pulp becomes inflamed. This hypothesis is supported by the published literature that claims ANGPT1 will induce anti-inflammatory effects through TEK-mediated signalling (Huang *et al.* 2010). We therefore expand our hypothesis to state that upon application of an orthodontic force, the pulp will become inflamed and induce an anti-inflammatory response through the up-regulation of TEK mRNA in order to regulate for these changes.

At 3 days of orthodontic force application there was a weak trend towards decreased FGF2, KDR and SELE protein abundances which matched those trends observed in the variability corrected mRNA data. The decrease in the abundance of SELE is linked in the literature to the anti-inflammatory response of TEK (Huang *et al.* 2010) but no anti-inflammatory response has been linked to decreased FGF2 or KDR protein expression patterns. We previously hypothesised that at 3 days of orthodontic treatment, the pulps are in a hyperoxic state in order to recover from inflammation induced hypoxia; when examining the trends in protein abundance, we found supporting evidence for our hypothesis in the literature (Asikainen *et al.* 2005, Hosford and Olson 2003, Lange *et al.* 2007, Sukhotnik *et al.* 2008, Valter *et al.* 1998) where hyperoxia was shown to decrease the abundance of FGF2, KDR and SELE analytes. It should be noted that the incidence of hyperoxia was thought to be repudiated due to the observed increase of PDGFB at this experimental time-point. We could find no evidence in the literature to link an

increase in PDGFB, and potentially increased interstitial fluid pressure, to a decreased abundance of FGF2, KDR and SELE.

At 2 weeks of treatment there were strong trends towards increased expression of FGF2 and SELE with a decreased expression of PDGFA. This trend did not match that of the mRNA expression analysis following correction for biological variability. It was observed at the mRNA level that FGF2 and PDGFA increased while there was no change between the control and treated states for SELE expression. When analysed by STRING (www.string-db.org) there was shown to be no link in signalling pathways or in the literature between FGF2 and SELE. FGF2 has been shown to up-regulate the cell adhesion molecules for SELE in inflamed tissues but no direct link has been found between increased FGF2 and SELE protein expression (Zittermann and Issekutz 2006). As the cell adhesion molecules had not been investigated in our study, we propose that further investigation be implemented in order to determine whether or not their expression is increased in our experimental model; thus indicating whether or not the pulps are in an inflammatory state at this time-point of orthodontic treatment. We also suggest that the occurrence of inflammation be examined across the full time-course of treatment to determine whether a cycle of inflammation and recovery is occurring in this tissue; and at what stage of tooth movement this inflammation is experienced by the tissue. The decrease in PDGFA protein abundance has been observed in hypoxia (Heinzman *et al.* 2008) alongside an increase in FGF2 (Heinzman *et al.* 2008, Silpanisong and Pearce 2013). A combination of both hypoxia and inflammation was shown to induce SELE expression (Zund G *et al.* 1996), suggesting that our hypothesis of hypoxia occurring at two weeks of force application, either as a response to or resulting in inflammation is supported by the literature.

4.5.11 Linking Protein Expression to Phenotypic Factors

In order to determine whether patient phenotype had any effect on protein expression, despite this being ruled out at the transcriptomic level; a qualitative analysis was performed on the data in collaboration with Dr Nora Donaldson. As per our findings in the mRNA expression analysis, the trends observed in protein expression amongst the time-points and between patients could not be conclusively linked to a phenotypic factor. At 1 day of force application Patient 3 and Patient 4 were linked by asian ethnicity, female gender and a closed apex morphology. When genes among these two patients were assessed for correlation there was found to be an increase in FGF2 and TEK proteins. Patients 2 and 5 were shown to be linked by black ethnicity, female gender and closed apex morphology but when the analyte expression of each of these patients were compared, no common expression could be determined. There was a general trend towards increased expression of FGF2, SELE and TEK although this could not be linked to a single phenotype across all patients.

At 3 days of force application Patient 2 and 3 in this group were shown to be linked by black ethnicity, female gender and a closed apex morphology although Patient 2 showed a general decrease in analyte expression relative to intra-patient controls and Patient 3 showed an increase in analyte concentration. The analytes for FGF2, KDR and SELE were all shown to be decreased in concentration at this time-point of treatment but there was no common phenotype which provided a link between protein expression patterns in these patients.

At 14 days of force application, Patients 1 and 3 from this group were linked by black ethnicity, female gender and open apex morphology. These two patients were linked to an increase in FGF2 protein expression and a decrease in PDGFA protein expression. When the rest of the patients in this group were compared, we observed an increase in FGF2 and SELE analyte concentrations and a decrease in PDGFA analyte

concentration; although no common phenotype could be found to link the protein expression patterns amongst these patients.

Despite the lack of conclusive evidence available at the mRNA level, we had maintained the hypothesis that tooth morphology would have a significant and linking effect on protein expression. This was not found to be the case and we suggest that in order to make a conclusive remark as to the effect of apex morphology on the pulp's response to routine orthodontic treatment; a substantially larger group of patients should be considered with both open and closed apices and the expression of genes should be determined at the transcriptomic and proteomic levels.

4.5.12 Linking mRNA Expression to Protein Expression

In order to determine whether the changes we observed as a response to orthodontic force application were consistent at both the transcriptomic and proteomic levels amongst the experimental time-points of treatment, normalised C_q values (**Table 58 – Table 60**) were compared to analyte concentrations (**Table 68 – Table 70**) from the multiplex ELISA and samples from the same patient were paired for comparison. Both sets of data were not corrected for biological variability due to the mRNA data correction step requiring the mean-centring of all data points and there being no methodology defined for ELISA data at this stage. Numerous genes were expressed in the same direction at both the transcriptomic and proteomic levels within a single patient, confirming that true changes in gene expression were occurring in the pulp as a response to routine orthodontic treatment. However, gene expression patterns and the direction of expression varied greatly amongst the samples within a single treatment time-point. It was anticipated that the observable differences in gene expression would be similar within a single treatment time-point but due to the inability to correct for biological variability and the inability to determine a baseline expression level for each

GOI, our findings are unsurprising. The differences in the expression of the GOIs observed within each sample group cannot be discussed in detail at this time, as each patient appears to react to force application with an individualistic response. Had a baseline been determinable, we would assume that any trends present would be more clearly observable.

For the genes which did not increase or decrease synchronically at the transcriptomic and proteomic levels, we can either attribute these differences to the lack of analyte abundance data available; or we can attribute them to a lack of correlation between mRNA expression and protein expression within the tissues themselves. As translation is the expected linking process between mRNA expression and protein abundance, we had expected a direct correlation between the two; this was not found to be true in our study as a number of the proteins of interest displayed an opposing or slightly varied mRNA expression pattern, suggesting that protein abundances are independent of mRNA expression. According to a publication by Yu *et al.* (2007) only a small number of studies have attempted to correlate mRNA expression levels with protein abundances; with a limited number of cases of correlation being reported. This suggests that complicated post-transcriptional and/or post-translational mechanisms are involved in gene regulation. According to a publication by Vogel and Marcotte (2012), approximately 40 % of the variation of protein concentration within cells is due to mRNA abundance whilst the remaining 60 % is ascribed to post-transcriptional regulation. Wang *et al.* substantiate this statement by commenting that processes such as mRNA degradation and stabilisation play a significant role in the protein abundance of the cell, as mRNA abundance is not only dependent upon the rate of transcription but also upon the rate of decay and degradation (Ing *et al.* 2005, Wang *et al.* 2002).

In this chapter, we have attempted to correlate mRNA expression patterns with those of protein expression without taking into consideration the regulatory mechanisms of both

the transcriptome and the pre-proteome. We have not considered the half-lives of the transcripts or the enzymes required to stabilise them, nor have we considered the small interfering RNA's (siRNA) or micro RNA's (miRNA) which attract endonucleases for cleavage or repress translation of mRNA, respectively. In order to further substantiate our findings and to attempt to link these findings to those already published by groups such as Derringer *et al.*, we propose that further work is carried out in order to determine the mechanisms by which stabilisation and translation of our GOIs occurs; specifically for those GOIs where we have seen significant changes in mRNA expression and clear trends amongst protein expression. However, particular caution should be taken when interpreting this data as these genes, enzymes, siRNA's and miRNA's are associated with multiple signalling cascades and making defined conclusions pertaining to cellular mechanisms of gene regulation is incredibly complicated.

In addition to this proposed further investigation of regulatory mechanisms, Yu *et al.* has designed a statistical tool to enable the correlation of mRNA and protein abundances using RT-qPCR and mass spectrometry data (Yu *et al.* 2007). We propose that the GOIs be examined by mass spectrometry and that the Protein Abundance and mRNA Expression (PARE) tool is utilised to attempt to determine if true correlation exists between the mRNA and proteins expressed within our treated samples.

4.6 Conclusion

In order to investigate the initial response of the human dental pulp to routine orthodontic treatment, in an attempt to determine the angiogenic response of the tissue to force application, we have increased the number of experimental time-points from a single investigation at 2 weeks to three separate time-points at 1, 3 and 14 days respectively and expanded our 'GOIs' panel from 4 to 17 genes. We have determined in this experimental chapter that:

1. The purchasing of optimised primers from a manufacturer, with guaranteed reaction efficiencies per primer pair, is far superior to optimising each primer set individually. This is particularly true with respect to smaller projects with limited time and financial resources available; as optimising primers and ensuring equal reaction efficiency can become incredibly costly when a large panel of genes are to be investigated.
2. Testing for primer specificity by using BLAST analysis and confirming the product sizes and binding efficiency by gel electrophoresis and dissociation or melt curve is essential to ensure that mRNA specific to the GOI is being amplified in the RT-qPCR reaction. Multiple genes may be amplified by a single primer set, and so the confirmation of the product size and the incidence of primer-dimer or spurious binding products should be assessed prior to performing RT-qPCR reactions on limited patient samples.
3. Biological variability has been shown to have a substantial effect on our experimental model and must be corrected for prior to performing relative expression analysis. Without correcting for biological variability, no trends could be observed between patients or between treatment groups.

4. In order to determine the source of biological variability amongst our samples, patient phenotypic factors such as age, gender, ethnicity and apex morphology were assessed and hypothesised to have a significant effect on the pulp's reaction to orthodontic force application. No statistically significant evidence could be determined from our sample group to support our hypothesis; however, the inflammatory response of the human dental pulp to orthodontic treatment needs to be considered further as apex morphology may have a potentially detrimental effect on this tissue under these conditions. Patient ethnicity was shown to have an effect on mRNA expression patterns but future work is required to elucidate the signalling cascades and translational mechanisms that differ between ethnicities.
5. It was shown, by regression analysis, that an application of orthodontic force had a significant effect on the expression patterns of a number of genes over the entire time-course of treatment, while other genes were shown to differ in expression pattern between two distinct time-points of treatment. We hypothesise that an application of routine orthodontic treatment at any point up to two weeks will result in hypoxia and subsequent angiogenesis in the human dental pulp. Between 1 and 3 days of force application there was a significant difference in the expression of genes associated with hypoxia and angiogenesis, suggesting that an application of orthodontic force will induce hypoxia and a subsequent angiogenic response. Between 3 days and 14 days, there was a change in expression of genes linked to hypoxia, angiogenesis and vascular differentiation. This suggested that in response to the initial oxidative stress, the tissue may be responding through angiogenesis and vascular differentiation. Between 1 and 14 days of force application, NOS2 was significantly altered in

expression, suggesting that through the entire duration of treatment, the pulp is in an inflammatory state.

6. By performing a factor analysis, MMP2, TGFB1 and VEGFA were shown to be the most tightly correlated genes under our experimental conditions which would suggest that pulps undergoing orthodontic force application undergo an amount of oxidative stress which results in inflammation and an angiogenic response to regulate for these changes. Tooth morphology may play a role at this stage and an inflammatory response may induce hypoxia and a subsequent corrective angiogenic response. Although, as the pro-inflammatory cytokines and chemokines IL8 and NOS2 were not shown to be correlated, perhaps this proposed hypothesis is irregular.
7. Willem *et al.*'s (2008) publication is sufficient to allow the extraction of statistically significant data from an otherwise undecipherable dataset due to the amount of variability within the control group. Following clarification of the methodology, which we have outlined in detail, we were able to determine statistically significant differences in mRNA expression between the control and treated groups at each experimental treatment time-point. Following 1 day of routine treatment, we determined that there was a change in mRNA expression associated with homeostasis of the vessel walls and a potential initiation of hypoxia in response to force application. Following 3 days of force application we found a general trend towards down-regulation of angiogenesis-specific mRNA and hypothesise that following 3 days of treatment the human dental pulp is in a state of both inflammation and hyperoxia, as a response to the original oxidative stress state seen at day 1. We suggest that these changes are attributed to tooth morphology, where most of the pulps investigated were extracted from teeth with closed apices, although we do acknowledge that

without correction for biological variability the impact of apex morphology on gene expression was found to be statistically insignificant. At 2 weeks of routine treatment, there was a statistically significant increase in the mRNA of HIF1A which is normally up-regulated solely in hypoxic conditions. This finding in conjunction with increased mRNA expression of genes associated with the coagulation process and inflammation might suggest that after 2 weeks of force application the tooth has attempted to recover from the initial inflammatory and hypoxic state, inducing angiogenesis and coagulation which has resulted in further inflammation and hypoxia due to the morphology of the tooth. These changes have yet to be confirmed by proteomic analysis and a more in-depth study of the time-points between those assessed in this study (day 2 and days 4 – 14) need to be considered. We propose that a full time-course from 1 day through to 21 days of treatment be considered as the studies by Derringer *et al.* (Derringer *et al.* 1996, Derringer and Linden 1998, Derringer and Linden 2003, Derringer and Linden 2004, Derringer and Linden 2007) treated teeth with force application for 14 days but then transferred the tissue to co-culture for an additional length of time, allowing for changes in mRNA and protein expression to continue prior to analysis.

8. The levels of sensitivity within the custom multiplex ELISA assay were too high to quantify the small analyte concentrations of the GOIs present in the human dental pulp samples. We propose that an alternate high-sensitivity assay for low-abundance proteins be used in order to assess these samples and obtain data that can then be corrected for biological variability, prior to drawing conclusions as to the pulp's response to orthodontic treatment.
9. When qualitatively assessed, our protein abundance data indicated that in response to orthodontic force application human dental pulps experience

inflammation, possible hyperoxia, hypoxia and angiogenesis. However, this data needs to be corroborated by further analysis where techniques such as light microscopy, *in situ* hybridisation and FACTT are used in a strict time-course on a much larger sample set.

10. The findings from our protein analyses do not negate the observations of Derringer *et al.* where an increase in secreted angiogenic growth factors was identified using blocking antibodies in co-culture of treated human dental pulps and rat aorta. Our findings either support Derringer *et al.*'s work, with specific reference to increased FGF2 and PDGFB abundances, or require further investigation in order to substantiate their experimental findings. As our quantification method had limited sensitivity and could not determine concentrations of low-level protein expression, we cannot dismiss their findings outright and propose that FACTT be used to quantify protein abundances in human dental pulp homogenates.
11. When relative mRNA expression data and analyte abundances were compared, few of the GOIs demonstrated a correlated expression pattern. However, the majority of the genes did not demonstrate any correlation between mRNA and analyte abundances. It has been determined that regulatory mechanisms in post-transcriptional and/or post-translational modification are crucial to the understanding of the conversion of increased mRNA to increased protein abundances; and that investigation of these regulatory mechanisms will elucidate the true changes within human dental pulps treated with an orthodontic force for a period of up to two weeks.

This experimental chapter has highlighted the importance of identifying correct protocols for statistical analysis of RT-qPCR data and low abundance protein concentrations present in human dental pulp homogenates. In it we have identified an

increased mRNA and protein expression of several angiogenesis-specific genes; and have suggested that following the application of orthodontic force, the pulp will experience inflammation, hypoxia and undergo angiogenesis in order to regulate for these changes. These responses to force application need to be investigated further.

Chapter V

Effects of Hypoxia on the Angiogenic Response in Human Dental Pulp Explants

5.1 Introduction

Hypoxia has been defined as the state where oxygen concentrations within a tissue or cell have been reduced below physiological levels, despite the presence of adequate perfusion by blood (Dorland 2007). It can occur continuously or intermittently whilst ranging in severity from an acute to a chronic condition, depending on the physiological or pathological cause. Hypoxia is mediated by the stabilisation of the mRNA of the transcription factor HIF1A and is regulated homeostatically by oxygen dependent hydroxylation of the ODD domain of the protein by PHD enzymes (Ivan *et al.* 2001, Jaakkola *et al.* 2001); or polyubiquitylation of the von Hippel-Lindau tumour suppressor (pVHL) E3 ligase complex (Jaakkola *et al.* 2001, Maxwell *et al.* 1999, Semenza 2003). Should the HIF1A transcript become stabilised by: (a) decreased oxygen concentration and an absence of oxygen-dependent hydroxylation, (b) nitrosylation of the oxygen-dependent domain of the HIF1A subunit by free radicals (Li *et al.* 2007) or (3) up-regulation of growth factors which inhibit PHD enzymes necessary for hydroxylation (Dengler *et al.* 2014, McMahon *et al.* 2006); angiogenesis, and the concordant up-regulation of angiogenesis-specific growth factor mRNA and increased protein transcription occurs in order to regulate oxygen tension (Krock *et al.* 2011, Pugh and Ratcliffe 2003, Semenza 2001).

Angiogenesis is the process of new vessel formation from a pre-existing capillary plexus (Brown *et al.* 1992, Li *et al.* 2003, Yano *et al.* 2003); involves a complex modification of the existing endothelium and is tightly regulated by a large number of cytokines, chemokines and growth factors.

Endothelial cells form the inner lining of all blood and lymph vessels within the body and are known to vary in phenotype between arterial and venous cells, and to vary throughout different portions of the vascular network (Sumpio *et al.* 2002).

Human dental pulp has been shown to be a highly vascular tissue, containing a dense capillary network which pervades the entire organ (Vongsavan and Matthews 1992, Yoshida and Ohshima 1996). Vasculature and lymph vessels enter the pulp through the apical foramina and from the trunk, the vessels begin to branch out toward the crown and the periphery; with the peripheral edge of the tooth containing the highest concentration of neovessels (Digka *et al.* 2006, Provenza 1958, Berggreen *et al.* 2009).

It has been shown in an *in vitro* study of cultured endothelial cells, that varying intensities of hypoxic exposure, namely: (a) intermittent hypoxia, (b) repeated cycles of intermittent hypoxia and (c) chronic hypoxia, will result in distinct transcriptomic and proteomic expression patterns between each exposure (Toffoli *et al.* 2009).

To date, hypoxia and the effects of various intensities of hypoxia have only been considered in the human dental pulp amongst *in vitro* cell lines in order to treat avulsed permanent teeth (Aranha *et al.* 2010), accelerate bone healing (Fujio *et al.* 2015.), increase the proliferation of stem cells (Sakdee *et al.* 2009.) and determine the mineralization response of the pulp during reparative dentinogenesis (Li *et al.* 2011). No previous studies have attempted to characterise the angiogenic response of the human dental pulp tissue treated with varying intensities of hypoxia, with respect to determining whether hypoxic intensity has a significant effect on neovessel development in this tissue.

As we have previously identified that an orthodontic force applied to teeth for two weeks prior to extraction may induce hypoxia (**Chapter 4**) in the human dental pulp, we propose that characterisation of the pulp's angiogenic response to varied intensities of hypoxia is critical for our elucidation of the true response to routine clinical orthodontic treatment. For this purpose, pulps from untreated teeth were exposed to a series of hypoxic exposures (chronic, intermittent and repeated cycles of intermittent hypoxia) as per Tofolli *et al.* (2009); and the transcriptomic and proteomic expression of

angiogenesis-specific growth factors was assessed. We propose an *a priori* hypothesis that pulps due to their dense vasculature, will respond to each intensity of hypoxia with a unique and distinct transcriptomic and proteomic expression pattern.

5.2 Aims

1. Expose untreated human dental pulps to a series of hypoxic intensities in order to characterise the pulp's angiogenic response to:
 - (a) intermittent hypoxia
 - (b) repeated cycles of intermittent hypoxia
 - (c) chronic hypoxia
2. Following exposure to hypoxia, incubate *ex vivo* human dental pulps under normoxic conditions *in vitro* for an extended period to determine the effect of a prolonged incubation period on the proteomic expression profile.
3. Compare the prolonged incubation protein expression profile to that of pulps snap-frozen immediately following exposure to hypoxia.

5.3 Materials and Methods

MATERIALS

5.3.1 Human Dental Pulp

Following informed parental and patient consent, human dental pulps were obtained from healthy first and second premolar teeth extracted from volunteers aged between 12 – 16 years. All patients included in the study required extraction of a minimum of 4 position-paired premolar teeth in conjunction with fixed appliance orthodontic treatment. Ethical approval was granted by the Research Ethics Committee at Guy's Hospital and King's College Hospital.

METHODS

5.3.2 Tooth Extraction and Dental Pulp Isolation

In this experimental chapter, pulps were isolated from 20 'control' teeth (four teeth from five patients) where no orthodontic forces had been applied to teeth prior to extraction, in order to analyse the effect of varying exposures to hypoxia on the relative mRNA and protein expression of angiogenesis-specific genes in human dental pulps.

A further 8 'control' pulps (two teeth from four patients) were isolated in order to analyse the expression of angiogenesis-specific proteins following 16 hr normoxic, *in vitro* recovery periods from hypoxic exposure.

All premolar teeth were extracted under local anaesthesia; and each tooth was placed in an individual tube containing 20 ml of cold sterile saline solution. Teeth were sectioned immediately with a high-speed water-cooled diamond bur through the buccolingual aspect and dental pulps with a mean mass of ± 0.05 g ($n = 5$) were removed from the pulp chamber with sterile blunt instruments. Isolated pulps were then individually

placed in sterile 7 ml bijoux tubes containing 5 ml Dulbecco's MEM (Cat. No. D6046) (Sigma-Aldrich, Poole, UK) supplemented with 50 U/ml penicillin (Cat. No. P4333) (Sigma-Aldrich, Poole, UK) and 50 µg/ml streptomycin (Cat. No. P4333) (Sigma-Aldrich, Poole, UK). Samples were placed on ice and transferred immediately to a laminar flow cabinet for sectioning under sterile conditions.

No orthodontic procedures were performed on these teeth prior to extraction. All dental pulp isolations, following extraction, were performed by Dr K. A. Derringer, Dental Institute, King's College London at Guy's Hospital and King's College Hospital.

5.3.3 Exposure of Human Dental Pulps to Hypoxia

Pulp samples were sectioned through the mid-sagittal plane with a sterile surgical scalpel and both pulp halves were transferred directly to an upright 25 cm² BD Falcon™ cell culture flask (Cat. No. 734-0009) (canted neck, plug-seal lids; VWR International Ltd., West Sussex, UK) containing 5 ml Dulbecco's MEM (Cat. No. D6046) (Sigma-Aldrich, Poole, UK) supplemented with 50 U/ml penicillin (Cat. No. P4333) (Sigma-Aldrich, Poole, UK) and 50 µg/ml streptomycin (Cat. No. P4333) (Sigma-Aldrich, Poole, UK).

Caps were removed from the tissue culture flasks containing supplemented medium and mid-sagittally sectioned pulps (Cat. No. 734-0009) (VWR International Ltd., West Sussex, UK) and pierced with a sterile 21G needle. Punctured lids were placed back onto flasks loosely, without tightening completely and a fresh, sterile 21G needle was slid gently into the hole in the punctured lid (**Figure 20**). Tissue culture flasks were flushed for 2 min with a 1 % O₂, 5 % CO₂, 94 % N₂ gas mix (BOC Special Gases, London, UK) at 5 psi, sealed tightly and needle ends were sealed with luer plugs. Culture flasks under hypoxia were incubated for differing lengths of time before releasing the seal and allowing tissue media to equilibrate with ambient oxygen and

5 % CO₂ for 30 min (see **Table 75** for details). Pulps from the same dental position in different patients were used for appointed hypoxic exposures. Pulp halves for protein analysis were recovered from flasks, placed in 1 ml cryotubes (Cat. No. 374503) (Nunc™, ThermoFisher Scientific, Nottingham, UK), snap-frozen in liquid nitrogen and transferred to – 70 °C for permanent storage. Pulp halves for mRNA analysis were recovered from flasks, immersed separately in 500 µl RNALater™ (Cat. No. AM7021) (Ambion Inc., Warrington, UK), an RNA preservation solution, kept at 4 °C for 8 hr and transferred to -20 °C for permanent storage.

Pulp halves for the *in vitro* recovery experiments were allowed to recover under normoxic conditions (ambient oxygen and 5 % CO₂) for a further 16 hr following equilibration, prior to snap-freezing, to allow for protein production; according to a modification of the protocol outlined by Tofolli *et al.* (2009) (see **Table 76** for details).

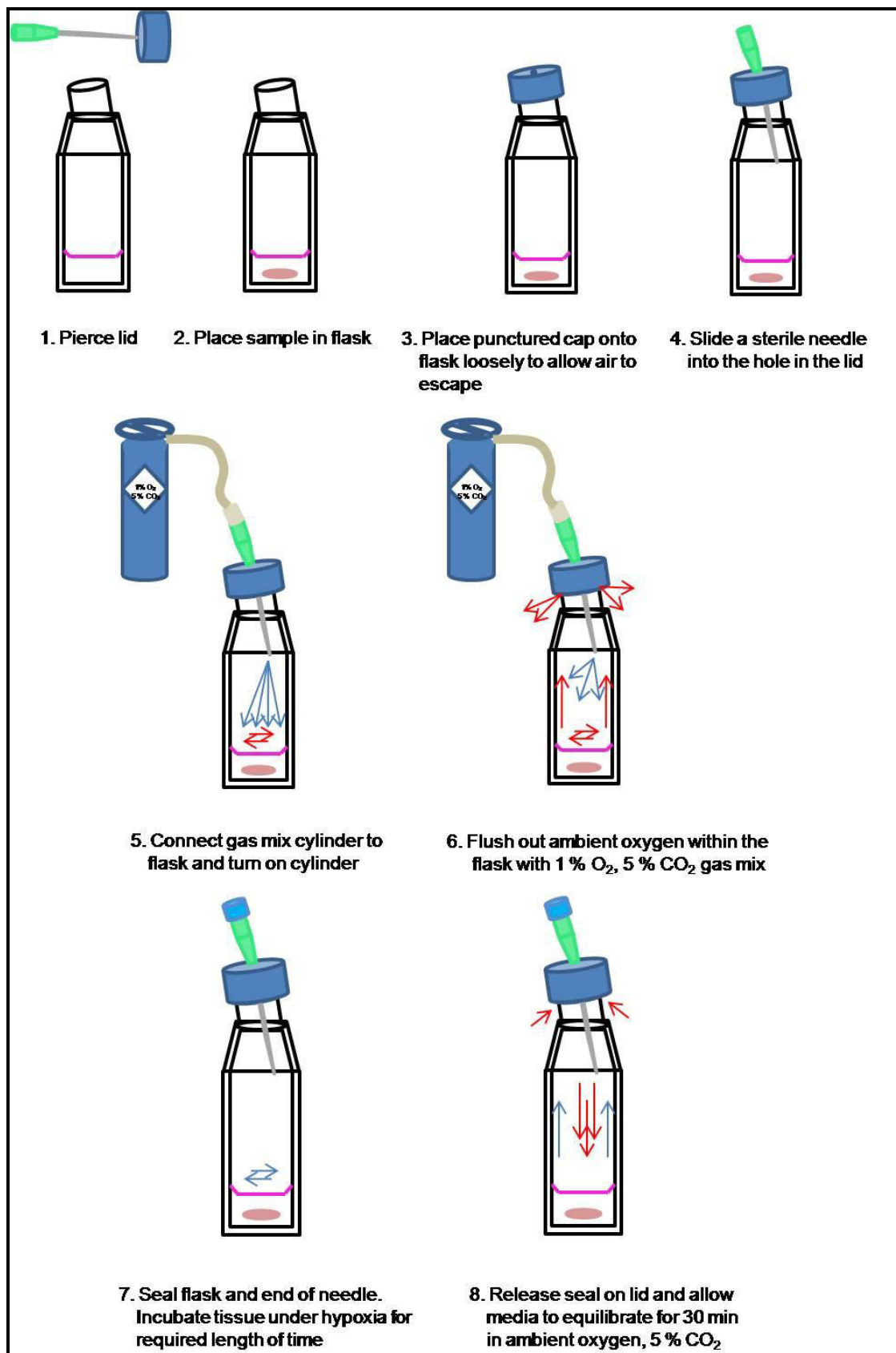


Figure 20 Inducing chronic and intermittent hypoxia in the human dental pulp

	Tooth Position:	Upper Right	Lower Right	Upper Left	Lower Left
	Hypoxic Exposure:	Control	Chronic Hypoxia	Intermittent Hypoxia	Repeated Intermittent Hypoxia
Exposure Protocol	Flush:	None	2 min	2 min	2 min
	Flush Conditions:	None	1% O ₂ , 5% CO ₂ & 94% N ₂	1% O ₂ , 5% CO ₂ & 94% N ₂	1% O ₂ , 5% CO ₂ & 94% N ₂
	Incubation:	5 h 30 min	5 h	1 h	1 h
	Incubation Conditions:	Ambient Oxygen with 5% CO ₂	Flask Sealed	Flask Sealed	Flask Sealed
	Equilibration:	None	30 min	30 min	30 min
	Equilibration Conditions:	None	Ambient Oxygen with 5% CO ₂	Ambient Oxygen with 5% CO ₂	Ambient Oxygen with 5% CO ₂ **Repeat Full Protocol 2 More Times**

Table 75 Protocols for induction of hypoxia in the human dental pulp

	Tooth Position:	Upper Right	Upper Left
	Hypoxic Exposure:	Control	Chronic Hypoxia
Exposure Protocol	Flush:	None	2 min
	Flush Conditions:	None	1% O ₂ , 5% CO ₂ & 94% N ₂
	Incubation:	5 h 30 min	5 h
	Incubation Conditions:	Ambient Oxygen with 5% CO ₂	Flask Sealed
	Equilibration:	None	30 min
	Equilibration Conditions:	None	Ambient Oxygen with 5% CO ₂
	Protein Incubation:	16 h	16 h
	Protein Incubation Conditions:	Ambient Oxygen with 5% CO ₂	Ambient Oxygen with 5% CO ₂

Table 76 Hypoxia protocols with long recovery periods for protein production

5.3.4 Validation of Hypoxic Exposure

Once samples had completed the flush, incubate, equilibrate cycle; 5 ml sample media was collected and transferred to a sterile 7 ml bijoux tube. Oxygen concentration (ppm and %) was measured for 5 min with the ELIT 9701a Aqualyser and Galvanic dissolved oxygen probe (NICO2000 Ltd., Middlesex, UK).

Sample readings were adjusted according to readings taken from pulp-free hypoxic media with a Fibre Optic Oxygen (FOXY) Probe (Ocean Optics, Florida, USA) at University College London in the laboratory of Prof. T. Arnett in collaboration with Ms. S. Taylor.

5.3.5 RNA Isolation

Preserved pulp samples were ground individually in a sterile, autoclaved 7 ml glass Dounce tissue homogeniser (Wheaton Science Products from VWR, Poole, UK) containing 600 µl of the RNA stabilisation solution RNA-Bee™ (Cat. No. CS-104B or CS-105B or CS-501B) (AMS Biotechnology, Abingdon, UK) and homogenates were transferred to sterile centrifuge tubes. 60 µl of chloroform (Cat. No. C7559) (Sigma-Aldrich, Poole, UK) was added to the homogenate and samples centrifuged at 4 °C for 15 min at 9,000 g. Supernatants were collected in fresh tubes and a single volume of 70 % ethanol was added. Samples were inverted repeatedly and transferred to spin columns and RNA_{total} extracted using the RNEasy Mini Kit (Cat. No. 74104 or 74106) (Qiagen Ltd., Crawley, UK) according to manufacturer's instructions. Isolated RNA_{total} was suspended in a final volume of 30 µl nuclease-free water (Cat. No. AM9938) (Ambion Inc., Warrington, UK) and stored at -70 °C.

5.3.6 RNA Quantification

The purity and concentration (ng/μl) of RNA_{total} for each sample was determined by calculating the A₂₆₀:A₂₈₀ and A₂₃₀:A₂₆₀ ratios using the NanoDrop™ 1000 spectrophotometer (Now replaced by Cat. No. ND-2000, NanoDrop™ 2000 spectrophotometer) (ThermoFisher Scientific, Nottingham, UK), according to manufacturer's instructions. RNA_{total} was then quantified by fluorescence with the Quant-iT™ Ribogreen® RNA Assay Kit (Cat. No. R11490) (Molecular Probes, Invitrogen Ltd., Paisley, UK), according to the manufacturer's protocol.

5.3.7 Reverse-Transcription

50 ng RNA_{total} was converted to cDNA using the WT-Ovation™ Pico RNA Amplification System (Now replaced by Cat. No. 3302, Ovation Pico WTA System V2) (NuGen Technologies, Inc., California, USA) according to the manufacturer's protocol. cDNA was then purified using the DNA Clean and Concentrator 25™ kit (Cat. No. D4033 or D4034) (Zymo Research Corporation, California, USA) according to manufacturer's instructions; and quantified with the NanoDrop™ 1000 spectrophotometer. Samples were stored permanently at – 20 °C.

5.3.8 RT-qPCR Analysis of mRNA Expression

RT-qPCR was performed on 20 μl reactions set up manually in 0.1 ml strip tubes (Cat. No. 981103) (Qiagen Ltd., Crawley, UK): 5 μl cDNA (2 ng/μl), 1 μl forward and reverse primer mix (6 μM) (designed and optimised by PrimerDesign Ltd., Southampton, UK), 10 μl Precision Mastermix with SYBR Green (Now replaced by Cat. No. PrecisionPlus-SY, PrecisionPlus™ Mastermix) (PrimerDesign Ltd., Southampton, UK) and 4 μl RNase/DNase-free water (delivered in conjunction with the primers, no individual Cat. No.) (PrimerDesign Ltd., Southampton, UK) with the

Corbett Rotor-Gene™ 6000 (No Cat. No. available but item is still available from Qiagen) (Corbett Life Science, Qiagen Ltd., Crawley, UK). Amplifications were performed with an initial template denaturation step at 95 °C for 10 min followed by 50 cycles of denaturation at 95 °C for 15 sec; combined primer annealing/extension at 60 °C for 60 sec; and data acquisition.

A disassociation protocol was performed from 72 °C to 95 °C at 1 °C increments in order to determine the presence of primer-dimer formation or other spurious binding products.

Details of primers are given in **Chapter 4, section 4.3.7.**

5.3.9 Statistical Analysis of RT-qPCR Data

Raw RT-qPCR data was analysed with Corbett Rotor-Gene™ 6000 Application Software, version 1.7 (Build 87) and expressed as quantification cycle (C_q) values. All C_q values of unknowns fell within the linear quantifiable range of the reaction. Reaction efficiencies were assumed to be 100%, as guaranteed by PrimerDesign Ltd., Southampton, UK

In order to eliminate the effect of biological variability amongst individuals on the average mRNA expression values, C_q values were analysed by the statistical tests outlined in ‘Standardization of real-time PCR gene expression data from independent biological replicates’ (Willems *et al.* 2008); namely log transformation, mean centring and autoscaling; where methodology was previously clarified by Dr Nora Donaldson, Statistical Advisor, Dental Institute, King’s College London. Data was normalised against the geometric mean expression of the reference genes RPL13A and UBC, previously established to be the most stably expressed pair of reference genes in our experimental system.

In order to identify patterns to determine whether patient ethnicity, gender or root morphology was linked to gene expression profiles, data was sent to Dr Nora Donaldson, Statistical Advisor, Dental Institute, King's College London for analysis. Statistical significance was given at $p \leq 0.05$.

5.3.10 Pulp Tissue Homogenisation for Protein Analysis

Individual snap-frozen pulp samples were homogenised in 2 ml sterile centrifuge tubes containing 250 μ l lysis buffer (Cat. No. EL-lysis) (RayBiotech Inc., Georgia, USA) supplemented with 25 μ l protease inhibitor cocktail (Cat. No. 539131) (Calbiochem®, Merck4Biosciences, Darmstadt, Germany) with an Ultra-Turrax® homogeniser (Cat. No. 3370100) (Rose Scientific Ltd., Alberta, Canada). Tissue lysates were centrifuged at 4 °C for 10 min at 5,000 g to separate tissue debris from supernatants; debris pellets were discarded and tissue lysate supernatants were stored on ice.

The total protein concentration was determined with the Pierce BCA Protein Assay Kit (Cat. No. 23250) (ThermoFisher Scientific, Essex, UK), according to manufacturer's instructions.

Samples were diluted to a concentration of 2 μ g/ μ l with lysis buffer supplemented with protease inhibitor cocktail (1:10 dilution) as per instruction from RayBiotech Inc., Georgia, USA before snap-freezing 200 μ l aliquots in liquid nitrogen and transferring to -70 °C for permanent storage.

5.3.11 Quantification of Proteins Associated with Angiogenesis

Frozen human dental pulp lysates (200 μ l at a concentration of 2 μ g/ μ l, with the exception of 1 sample of 150 μ l at a concentration of 0.244 μ g/ μ l) were delivered to RayBiotech Inc., Georgia, USA for Quantibody® Array Testing. 12.5 μ l of the human dental pulp lysate was added to each well of a custom Quantibody® multiplex sandwich-based ELISA in addition to 12.5 μ l running buffer, making a total

concentration of 1 $\mu\text{g}/\mu\text{l}$ (with the exception of a single sample which held a final concentration of 0.122 $\mu\text{g}/\mu\text{l}$). Reactions were performed in technical quadruplicate. The custom Quantibody® ELISA array targeted the following angiogenesis associated proteins: ANGPT1; FGF2; KDR; MMP2; PDGFA; PDGFB; SELE; TEK; TGFB1; VEGFA. Analyte concentrations were determined and delivered in a service report.

5.4 Results

5.4.1 Verification of Hypoxic Exposure by Measuring Oxygen Tension

In order to determine the total percentage of oxygen to which the human dental pulp explants would be exposed during the hypoxia experiments; sterile culture media was analysed with an ELIT 9701a Aqualyser and a Galvanic dissolved oxygen probe following exposure to control, intermittent, repeated intermittent and chronic hypoxia conditions as outlined in **Table 75** and **Table 76**. In order to verify the oxygen concentrations calculated by the ELIT 9701a Aqualyser and Galvanic dissolved oxygen probe, experiments were repeated and oxygen concentrations were measured with a FOXY probe.

Due to the nature of the FOXY probe, the oxygen partial pressure, displayed as a total oxygen percentage, is measured in a photoluminescence-quenching reaction by a spectrometer based on a phase shift from a state of complete anoxia to hypoxia/normoxia/hyperoxia. Galvanic probes do not provide a direct reading; instead oxygen is measured in parts per million or percentages of atmospheric oxygen, which is taken to be 100 %.

In order to align the Galvanic probe readings with those of the FOXY probe, data from both probes was plotted on straight line graphs following an adjustment of the Galvanic probe data to the estimated percentage of oxygen present ambiently; we assumed that atmospheric oxygen concentrations ranged from 5 – 25 %. There was shown to be no direct correlation between the FOXY and Galvanic probes at any of the hypoxic exposures when a single adjustment to atmospheric oxygen was made, e.g. adjustment to 21 % atmospheric oxygen shown in **Figure 21**.

Instead, in order to adjust the Galvanic probe's readings to align with those observed with the FOXY probe, we had to assume that ambient oxygen concentration within control pulp media was 19 %. (**Figure 21**). After exposing the media to the hypoxia gas mix, for an intermittent period or to repeated intermittent hypoxia, the FOXY probe and Galvanic probe readings were the closest after an adjustment of the Galvanic probe data to an atmospheric oxygen concentration of 10% (**Figure 21**). When exposed to chronic hypoxia, similar readings between the FOXY probe and Galvanic probe were reached after an adjustment of the Galvanic probe data to 24 % atmospheric oxygen (**Figure 21**). For the purpose of verifying the hypoxic exposure of all human dental pulp explants, the Galvanic probe data was adjusted according to the line of best fit calculated based on data from the FOXY probe and the established ambient oxygen concentration (%) corrections.

It was shown that following adjustment to the line of best fit from the FOXY probe, that there were still discrepancies between the data collected from FOXY and Galvanic probes (**Table 77**). These discrepancies can be attributed to the way in which the FOXY and Galvanic probe data was collected. Data from the Galvanic probe (**Table 77**) was collected from media in which human dental pulp explants were immersed; the media was exposed to hypoxia and then allowed to equilibrate in ambient oxygen supplemented with 5 % CO₂ for 30 min prior to pulp isolation and measurement with the dissolved oxygen probe. Data from the FOXY probe was collected from pulp-free media without equilibration in supplemented ambient oxygen.

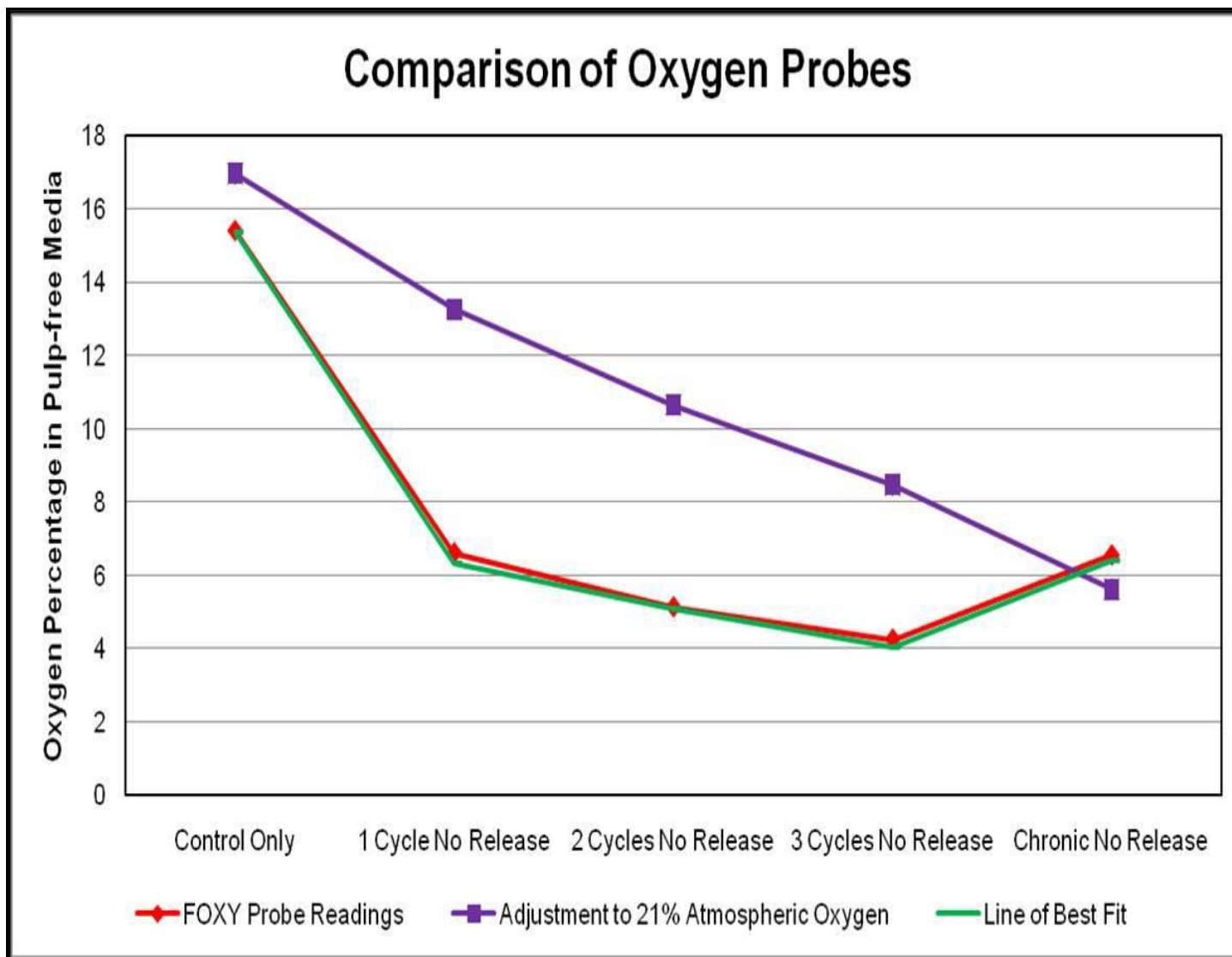


Figure 21 Comparison of Oxygen Probes for Validation of Hypoxia Exposure

Graph illustrating oxygen percentage of pulp-free media exposed to hypoxia as calculated by the FOXY probe (red line) and the Galvanic probe (purple and green lines). The purple line illustrates Galvanic probe data following an adjustment to 21 % atmospheric oxygen. The green line illustrates a line of best fit constructed by adjusting Galvanic probe data to 19 %, 10 % 10 %, 10 % and 24 % atmospheric oxygen respectively.

	Control	Intermittent Hypoxia	Repeated Intermittent Hypoxia	Chronic Hypoxia
FOXY Probe	15.40	6.60	4.24	6.54
Galvanic Probe	12.32	6.66	7.12	12.28

Table 77 Average Oxygen Concentration (%) in Culture Media Following Hypoxic Exposure

This table illustrates the discrepancies between FOXY and Galvanic probe readings following adjustment of Galvanic probe data to ambient oxygen percentages calculated by the line of best fit (**Figure 21**). Data from the FOXY probe was calculated from pulp-free media exposed to hypoxia without a recovery period of 30 min exposure to ambient oxygen supplemented with 5 % CO₂ between intermittent cycles or after hypoxic exposure. Data from the Galvanic probe was calculated from media which had contained human dental pulp explants, was exposed to hypoxia and was then allowed to recover for 30 min in supplemented ambient oxygen.

5.4.2 Statistical Standardisation of Biological Variability in RT-qPCR

Data and Relative Expression Analysis

RT-qPCR was performed in technical duplicate using primers for the angiogenesis-specific growth factors: ANGPT1, FGF2, HIF1A, IL8, ITGAV, ITGB3, KDR, MMP2, NOS2, PDGFA, PDGFB, ROBO4, SELE, TEK, TGFB1, TGFBR1 and VEGFA on human dental pulp explants exposed to no hypoxia (control) (n = 5), intermittent (n = 5), repeated intermittent (n = 5) and chronic (n = 5) hypoxic conditions (as outlined in **Table 75 & Table 76**). Raw RT-qPCR data was analysed with Corbett Rotor-Gene™ 6000 Application Software, version 1.7 (Build 87) and expressed as quantification cycle (C_q) values.

In order to eliminate the effect of biological variations between the individuals C_q values were log-transformed, mean centred and auto-scaled according to the methodology outlined by Willems *et al.* (2008). It was shown that following exposure to intermittent hypoxia, there was a statistically significant up-regulation of ANGPT1, MMP2, ROBO4, TGFB1 and TGFBR1 mRNA expression and a significant down-

regulation of IL8 and ITGB3 mRNA expression in treated samples relative to untreated controls (**Figure 22**).

Following repeated exposures to intermittent hypoxia there were no statistically significant differences in the mRNA expression of any of the GOIs (**Figure 23**).

There was a statistically significant up-regulation of HIF1A, IL8 and TGFBR1 mRNA expression in samples treated with chronic hypoxia compared with untreated controls (**Figure 24**).

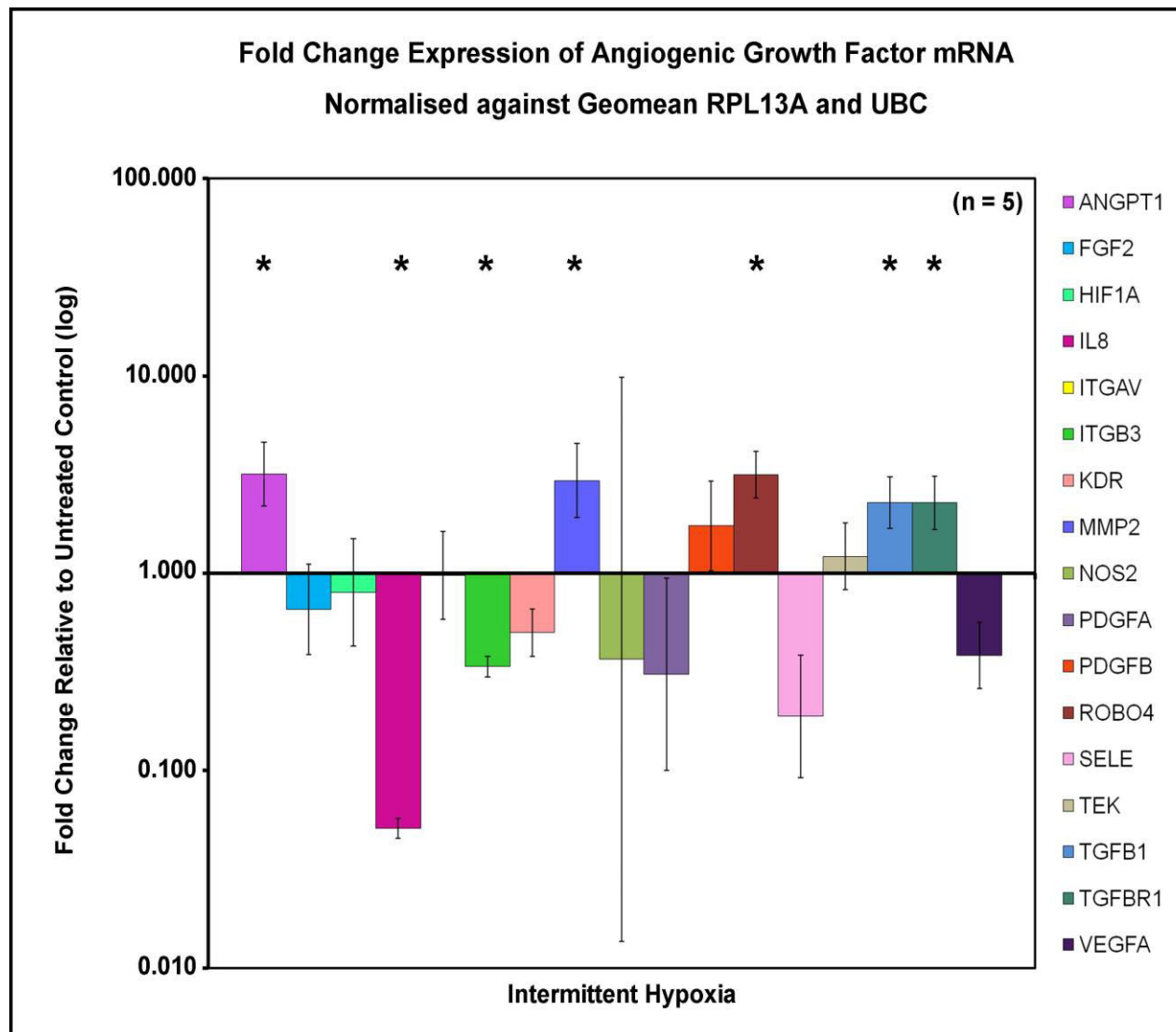


Figure 22 Fold change expression analysis of angiogenic growth factor mRNA in pulp explants exposed to intermittent hypoxia relative to normoxic controls. Normalising against the geometric mean of RPL13A and UBC

Plot shows the fold-change (log) of mRNA expression (columns); and the error of the mean (error bars) in hypoxically exposed pulp explants relative to normoxic controls. Data was normalised against the geometric mean of RPL13A and UBC. There was a significant up-regulation of ANGPT1, MMP2, ROBO4, TGFB1 and TGFBR1 mRNA expression; and a significant down-regulation of IL8 and ITGB3 mRNA expression in the explants exposed to intermittent hypoxia (n = 5) relative to normoxic control explants (n = 5).

* indicates statistical significance ($p < 0.05$).

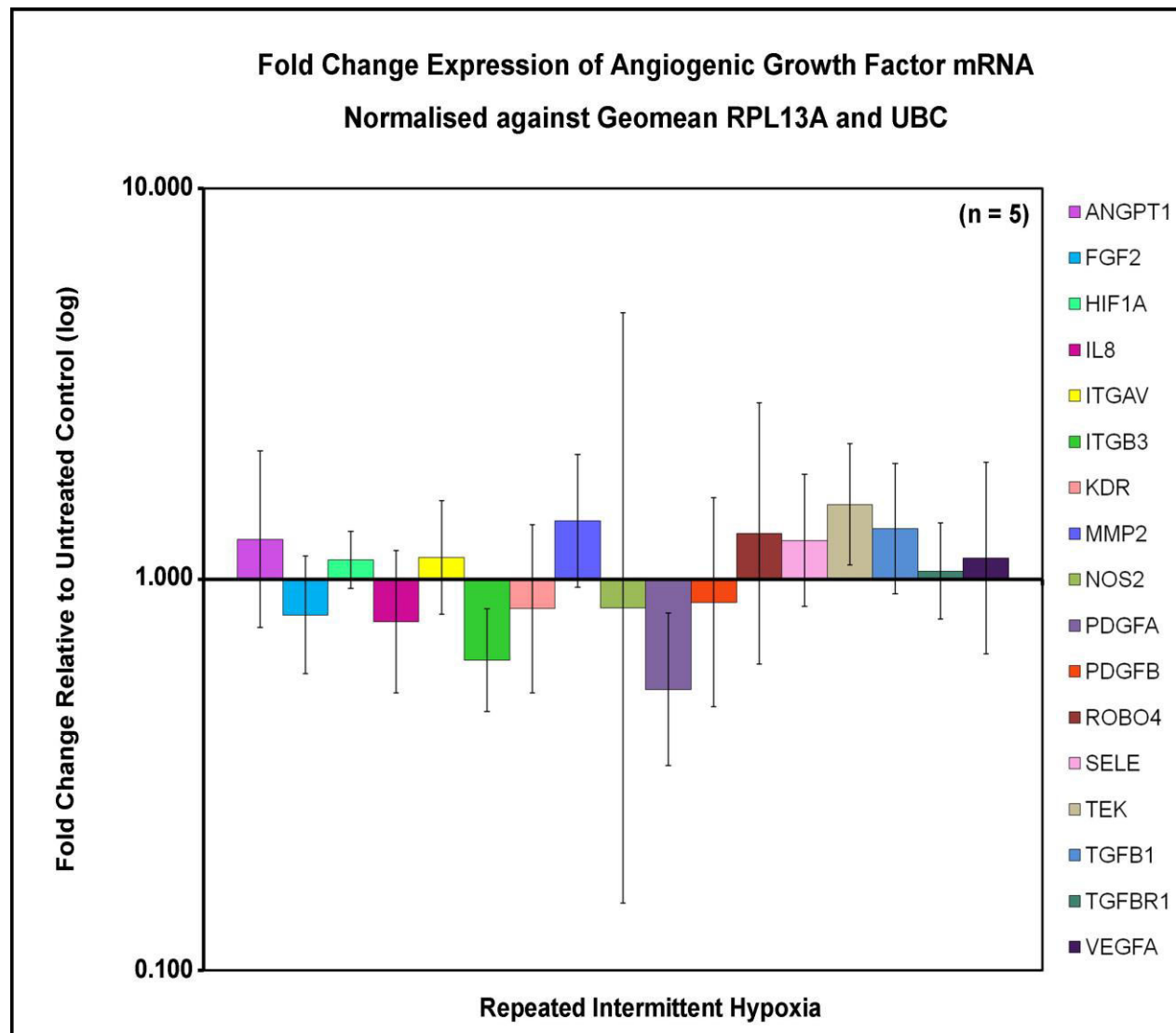


Figure 23 Fold change expression analysis of angiogenic growth factor mRNA in pulp explants exposed repeatedly to cycles of intermittent hypoxia relative to normoxic controls. Normalising against the geometric mean of RPL13A and UBC

Plot shows the fold-change (log) of mRNA expression (columns); and the error of the mean (error bars) in hypoxically exposed pulp explants relative to normoxic controls. Data was normalised against the geometric mean of RPL13A and UBC. There was no significant difference ($p > 0.05$) in mRNA expression of any of the angiogenic growth factors in explants treated with consecutive exposures to intermittent hypoxia ($n = 5$) relative to normoxic controls ($n = 5$).

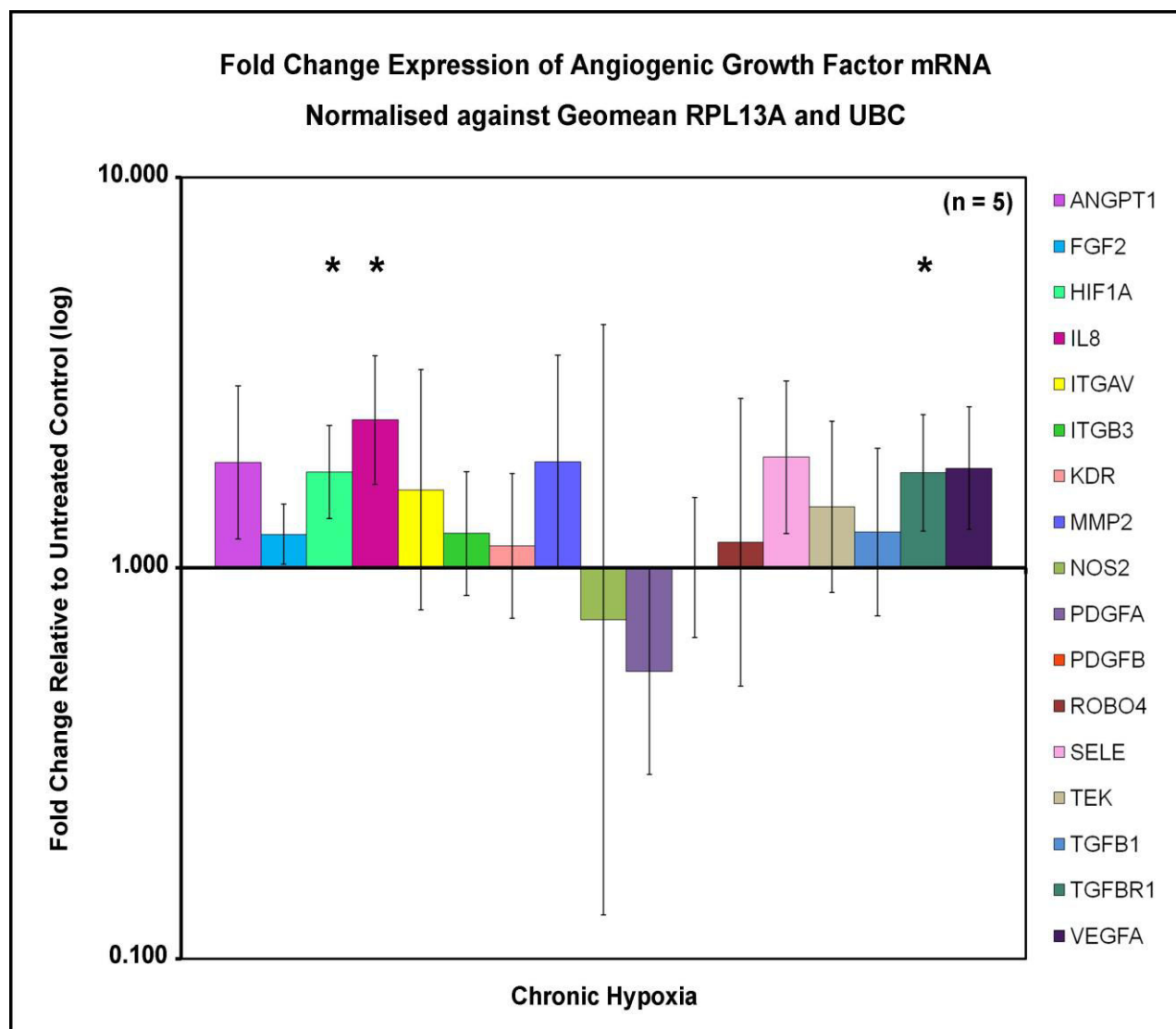


Figure 24 Fold change expression analysis of angiogenic growth factor mRNA in pulp explants exposed to chronic hypoxia relative to normoxic controls. Normalising against the geometric mean of RPL13A and UBC

Plot shows the fold-change (log) of mRNA expression (columns); and the error of the mean (error bars) in hypoxically exposed pulp explants relative to normoxic controls. Data was normalised against the geometric mean of RPL13A and UBC. There was a significant up-regulation of HIF1A, IL8 and TGFBR1 mRNA expression in the explants exposed to chronic hypoxia (n = 5) relative to normoxic control explants (n = 5).

* indicates statistical significance ($p < 0.05$).

5.4.3 The Effect of Phenotypic Factors on mRNA Expression Levels

In order to determine whether a patient's phenotypic characteristics i.e. ethnicity, gender and apical/apex morphology of the tooth from which the pulp was extracted played a specific role in mRNA expression patterning among the GOIs; patient details (see **Table 78**) were compared directly to trends observed in an analysis of inter- and intra-patient trends (**Chapter 8, section 8.3.3**).

In pulp explants exposed to intermittent hypoxia, we observed a trend of up-regulated mRNA expression in Patient 2. As there were no other patients exhibiting strong trends in mRNA expression, we could make no comparison of patient phenotype in this treatment group (**Chapter 8, section 8.3.3**).

As a response to repeated exposures to intermittent hypoxia, we found a common trend in down-regulation of mRNA expression amongst all patients (**Chapter 8, section 8.3.3**). When patient phenotype was analysed, we observed that 3/5 patients were white, 3/5 patients had a closed apex morphology and 3/5 patients were female. However, none of the patients shared the same combination of phenotypic traits and therefore, changes in mRNA expression could not be attributed to a specific phenotypic characteristic.

There was a trend of down-regulation in Patient 1 and Patient 5 following exposure of the human dental pulp explants to chronic hypoxia (**Chapter 8, section 8.3.3**). Evaluation of the phenotypes of these two patients indicates similar apex morphology but does not show mutual gender or ethnicity between the patients (**Table 78**).

As the patient size is small ($n = 5$), it would be inappropriate to make conclusive statements as to the effect of phenotype on gene expression in human dental pulp explants exposed to hypoxia.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Gender	F	M	F	F	M
Ethnicity	White	White	White	Black	Black
Apical Morphology	Closed	Open	Open	Closed	Closed

Table 78 Details of hypoxia patients' root morphology, ethnicity and gender

In order to gain a better understanding of the effect of phenotypic characteristics on each of the GOIs across all exposures to hypoxia, C_q values were normalised and a univariate linear regression analysis was carried out for each of the phenotypic factors, i.e. age, gender, ethnicity and apex morphology (**Chapter 8, section 8.3.4**). A summary table of the results of this analysis is given as **Table 79**. Significance was given at $p \leq 0.05$ whilst borderline significance was in the range of $0.12 \leq p < 0.05$.

It was shown that patient age had a significant effect on the mRNA expression of ANGPT1 and a borderline significant effect on the mRNA expression of ROBO4 (**Table 79**). Patient gender was shown to have a significant effect on the mRNA expression of MMP2 and TGFB1 (**Table 79**). ANGPT1, PDGFA and VEGFA mRNA expression was shown to be effected significantly by patient ethnicity. Patient ethnicity was also shown to have a borderline significant effect on FGF2 and TEK mRNA expression (**Table 79**). Apex morphology was shown to have no statistically significant effect on mRNA expression in any of the GOIs (**Table 79**).

Angiogenesis-Specific Growth Factor	Age	Gender	Ethnicity	Apex Morphology
ANGPT1	✓	✗	✓	✗
FGF2	✗	✗	BORDERLINE	✗
HIF1A	✗	✗	✗	✗
IL8	✗	✗	✗	✗
ITGAV	✗	✗	✗	✗
ITGB3	✗	✗	✗	✗
KDR	✗	✗	✗	✗
MMP2	✗	✓	✗	✗
NOS2	✗	✗	✗	✗
PDGFA	✗	✗	✓	✗
PDGFB	✗	✗	✗	✗
ROBO4	BORDERLINE	✗	✗	✗
SELE	✗	✗	✗	✗
TEK	✗	✗	BORDERLINE	✗
TGFB1	✗	✓	✗	✗
TGFB1	✗	✗	✗	✗
VEGFA	✗	✗	✓	✗

Table 79 Univariate linear regression analysis of patient phenotypes in hypoxically treated human dental pulp explants

5.4.4 The Effect of Hypoxic Intensity on mRNA Expression Levels

A mixed linear regression analysis was then performed in order to construct a one-way ANOVA test and pair-wise regression analysis for each of the GOIs. These tests were carried out in order to determine whether or not exposure of human dental pulp explants to any level of hypoxia would have a significant effect on mRNA expression; and to ascertain as to whether or not there were differences in the expression levels for each gene between two intensities of hypoxic exposure (**Chapter 8, section 8.3.5**). A summary table of the results of this analysis is given as **Table 80**. Significance was given at $p \leq 0.05$ whilst borderline significance was in the range of $0.12 \leq p < 0.05$.

It was shown that exposure to any intensity or duration of hypoxia had a significant effect on ANGPT1, FGF2, HIF1A, IL8, ITGB3, MMP2, ROBO4, SELE, TGFB1,

TGFBR1 and VEGFA mRNA expression levels; and a borderline significant effect on ITGAV, KDR and PDGFB mRNA expression (**Table 80**).

There was shown to be a significant difference in mRNA expression between control and intermittent hypoxia exposure in the following genes: ANGPT1, FGF2, IL8, ITGB3, MMP2, PDGFB, ROBO4, SELE, TGFB1, TGFBR1 and VEGFA; with a borderline significant difference in mRNA expression of KDR and PDGFA at the same level of exposure to hypoxia (**Table 80**).

Between control and repeated exposures to intermittent hypoxia, there was a significant difference in the mRNA expression of ITGB3 (**Table 80**).

It was shown that between control and chronic exposure to hypoxia, there was a significant difference in the expression of ANGPT1, HIF1A, IL8, ITGAV, MMP2 and TGFBR1 mRNA (**Table 80**).

Pair-wise regression analysis indicated a significant difference in mRNA expression of ANGPT1, IL8, ITGB3, KDR, MMP2, PDGFB, SELE, TGFB1, TGFBR1 and VEGFA; and a borderline significant difference in ROBO4 mRNA expression in pulp explants treated with repeated intermittent hypoxia relative to those exposed to intermittent hypoxia (**Table 80**).

There was a significant difference in mRNA expression of ANGPT1, FGF2, HIF1A, IL8, ITGAV, ITGB3, KDR, MMP2, PDGFB, ROBO4, SELE, TGFB1 and VEGFA in human dental pulp explants exposed to chronic hypoxia when compared to mRNA expression in pulp explants treated with intermittent hypoxia (**Table 80**).

Between chronic and repeated exposures to intermittent hypoxia, there was shown to be a significant difference in mRNA expression of ANGPT1, FGF2, HIF1A, IL8, ITGAV, ITGB3, TGFBR1 and VEGFA; there was also a borderline significant difference in KDR mRNA expression (**Table 80**).

Angiogenesis-Specific Growth Factor	Hypoxic Exposure	Control vs Intermittent Hypoxia	Control vs Repeated Intermittent Hypoxia	Control vs Chronic Hypoxia	Intermittent Hypoxia vs Repeated Intermittent Hypoxia	Intermittent Hypoxia vs Chronic Hypoxia	Repeated Intermittent Hypoxia vs Chronic Hypoxia
ANGPT1	✓	✓	✗	✓	✓	✓	✓
FGF2	✓	✓	✗	✗	✗	✓	✓
HIF1A	✓	✗	✗	✓	✗	✓	✓
IL8	✓	✓	✗	✓	✓	✓	✓
ITGAV	BORDERLINE	✗	✗	✓	✗	✓	✓
ITGB3	✓	✓	✓	✗	✓	✓	✓
KDR	BORDERLINE	BORDERLINE	✗	✗	✓	✓	BORDERLINE
MMP2	✓	✓	✗	✓	✓	✓	✗
NOS2	✗	✗	✗	✗	✗	✗	✗
PDGFA	✗	BORDERLINE	✗	✗	✗	✗	✗
PDGFB	BORDERLINE	✓	✗	✗	✓	✓	✗
ROBO4	✓	✓	✗	✗	BORDERLINE	✓	✗
SELE	✓	✓	✗	✗	✓	✓	✗
TEK	✗	✗	✗	✗	✗	✗	✗
TGFB1	✓	✓	✗	✗	✓	✓	✗
TGFBR1	✓	✓	✗	✓	✓	✗	✓
VEGFA	✓	✓	✗	✗	✓	✓	✓

Table 80 One-way ANOVA and pair-wise regression analysis of the effect of hypoxic exposure and intensity on mRNA expression levels

5.4.5 Protein Expression Profiles as a Response to Hypoxia

In order to identify the effects of different hypoxic exposures on the protein expression profiles of human dental pulp explants, a custom multiplex ELISA (Quantibody® Array) was performed by RayBiotech, Inc., USA

It was shown that for most of the angiogenesis-specific growth factors analysed, the level of analyte present in the standardised concentration of 2 µg/µl total protein, recommended by RayBiotech, Inc., was below the assay's lower LOD (**Table 81 – Table 83**). FGF2 was the only protein of interest analysed in which the concentration of analyte present in all samples under all experimental conditions was greater than the assay's lower LOD (**Table 81 – Table 83**).

Statistical analysis could not be performed on data from the protein assay, as calculated analyte concentrations were below the assay LOD and several samples gave a reading of 0 pg/ml. Upon qualitative examination of trends amongst individual patients in each hypoxia treatment group, by the exclusion of any patient pair where either the control or treated value was equal to 0 pg/ml, we noted that there were several differences in analyte abundance following hypoxic exposure. In human dental pulp explants exposed to intermittent hypoxia there was an increase in the abundances of MMP2, PDGFA, PDGFB, SELE and TEK. FGF2 analyte concentration was decreased in these explants, relative to control samples (**Table 81**). Following exposure to repeated intermittent hypoxia, there was trend towards increased FGF2 abundance and decreased PDGFB and TEK abundances (**Table 82**). After exposing pulp explants to chronic hypoxia there was a trend towards an increase in MMP2, PDGFA and SELE analyte concentration; with a decrease in FGF2, PDGFB and TEK (**Table 83**).

We also qualitatively assessed the inter-treatment trends and found there to be an increase in FGF2 and SELE analyte concentration, with a decrease in PDGFB between

the intermittent and repeated exposures to intermittent hypoxia. Between the repeated intermittent hypoxia and chronic hypoxia groups we observed an increase in KDR, PDGFB and TEK analyte concentration; and a decrease in FGF2, MMP2, PDGFA, SELE and VEGFA abundances. When comparing samples exposed to intermittent and chronic hypoxia, we observed a trend towards increased analyte concentration of KDR and SELE, with decreased abundances of FGF2, MMP2 and PDGFB.

GENES OF INTEREST	LIMIT OF DETECTION pg/ml	PATIENT 1		PATIENT 2		PATIENT 3		PATIENT 4		PATIENT 5	
		CONTROL	INTERMITTENT	CONTROL	INTERMITTENT	CONTROL	INTERMITTENT	CONTROL	INTERMITTENT	CONTROL	INTERMITTENT
ANGPT1	105.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FGF2	260.2	1765.8	1924.6	2187.5	909.4	2431.6	11540.7	1019.9	588.1	808.9	790.1
KDR	19.5	0.0	0.0	0.0	0.7	0.0	1.6	0.0	0.5	0.0	0.0
MMP2	301.9	0.0	0.0	130.9	0.0	233.3	504.9	119.0	0.0	0.0	0.0
PDGFA	23.8	2.7	3.7	1.9	11.9	4.3	18.5	16.7	3.4	0.0	0.0
PDGFB	2.9	1.4	1.7	0.3	0.8	4.9	9.5	6.6	0.0	1.4	0.0
SELE	67.2	89.4	0.0	81.3	118.4	39.6	18.4	64.4	75.7	8.1	37.2
TEK	64.6	23.1	0.0	13.4	28.7	67.7	76.4	76.3	29.0	19.7	0.0
TGFB1	444.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
VEGFA	7.9	0.0	0.0	0.0	0.0	0.0	2.0	0.0	0.0	0.0	0.0

Table 81 Analyte concentrations (pg/ml) of angiogenesis-specific GOIs in human dental pulp explants exposed to intermittent hypoxic conditions

Cells marked in blue represent the assay's lower LOD (pg/ml). Cells marked in purple represent samples that expressed an analyte concentration greater than the assay's lower LOD. Cells marked in white represent samples which were below the assay's LOD. Cells marked in green represent samples which did not express any detectable analyte concentration of the protein of interest.

GENES OF INTEREST	LIMIT OF DETECTION pg/ml	PATIENT 1		PATIENT 2		PATIENT 3		PATIENT 4		PATIENT 5	
		CONTROL	REP. INTERMITTENT	CONTROL	REP. INTERMITTENT	CONTROL	REP. INTERMITTENT	CONTROL	REP. INTERMITTENT	CONTROL	REP. INTERMITTENT
ANGPT1	105.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FGF2	260.2	1765.8	2057.7	2187.5	673.6	2431.6	5629.2	1019.9	1078.4	808.9	3130.9
KDR	19.5	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
MMP2	301.9	0.0	341.9	130.9	0.0	233.3	0.0	119.0	0.0	0.0	0.0
PDGFA	23.8	2.7	24.4	1.9	0.0	4.3	1.4	16.7	0.0	0.0	7.1
PDGFB	2.9	1.4	0.3	0.3	0.0	4.9	1.6	6.6	0.0	1.4	0.0
SELE	67.2	89.4	11.3	81.3	0.0	39.6	348.6	64.4	49.1	8.1	121.4
TEK	64.6	23.1	8.4	13.4	0.0	67.7	0.0	76.3	0.0	19.7	0.0
TGFB1	444.0	0.0	4.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
VEGFA	7.9	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 82 Analyte concentrations (pg/ml) of angiogenesis-specific GOIs in human dental pulp explants exposed to repeated intermittent hypoxic conditions

Cells marked in blue represent the assay's lower LOD (pg/ml). Cells marked in purple represent samples that expressed an analyte concentration greater than the assay's lower LOD. Cells marked in white represent samples which were below the assay's LOD. Cells marked in green represent samples which did not express any detectable analyte concentration of the protein of interest.

GENES OF INTEREST	LIMIT OF DETECTION pg/ml	PATIENT 1		PATIENT 2		PATIENT 3		PATIENT 4		PATIENT 5	
		CONTROL	CHRONIC	CONTROL	CHRONIC	CONTROL	CHRONIC	CONTROL	CHRONIC	CONTROL	CHRONIC
ANGPT1	105.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FGF2	260.2	1765.8	703.1	2187.5	815.9	2431.6	1568.9	1019.9	462.7	808.9	896.3
KDR	19.5	0.0	4.2	0.0	3.3	0.0	0.7	0.0	10.9	0.0	0.5
MMP2	301.9	0.0	328.5	130.9	0.0	233.3	355.0	119.0	0.0	0.0	698.2
PDGFA	23.8	2.7	10.0	1.9	0.0	4.3	11.3	16.7	0.0	0.0	3.3
PDGFB	2.9	1.4	2.2	0.3	0.7	4.9	1.9	6.6	1.5	1.4	0.8
SELE	67.2	89.4	90.4	81.3	52.6	39.6	78.7	64.4	0.0	8.1	116.0
TEK	64.6	23.1	21.5	13.4	0.0	67.7	4.1	76.3	41.5	19.7	0.0
TGFB1	444.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
VEGFA	7.9	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 83 Analyte concentrations (pg/ml) of angiogenesis-specific GOIs in human dental pulp explants exposed to chronic hypoxic conditions

Cells marked in blue represent the assay's lower LOD (pg/ml). Cells marked in purple represent samples that expressed an analyte concentration greater than the assay's lower LOD. Cells marked in white represent samples which were below the assay's LOD. Cells marked in green represent samples which did not express any detectable analyte concentration of the protein of interest.

5.4.6 Linking Patient Phenotypes to Protein Expression Profiles

In order to determine whether phenotypic factors such as ethnicity, gender and apex morphology played a specific role in the observed protein expression profiles; patient details (see **Section 5.4.3; Table 78**) were compared directly to trends identified in **Table 81 – Table 83**.

We previously observed no strong trends ($\geq 80\%$) in protein expression in explants treated with exposure to intermittent hypoxia (**Table 81**). Therefore we could not use patient phenotypes to explain any patterns in protein expression.

Following exposure to repeated intermittent hypoxia we observed a strong trend ($\geq 80\%$) toward increased expression of the FGF2 protein in the majority of patients (**Table 82**). Upon examination of patient phenotype (**Table 78**), it was noted that 3 of the 4 patients exhibiting an increase in FGF2 protein expression had a closed apex morphology; 2 patients were white while the other 2 were black; and 3 of the 4 patients were female (**Table 78; Table 82**). There was no direct correlation between the 3 patients with closed apex morphology and the 3 female patients.

After exposure to chronic hypoxia there was a trend of decreased FGF2 protein expression in treated explants relative to normoxic controls (**Table 83**). When patient phenotypes were observed in order to identify determining factors for this trend it was noted that 2 of the 4 patients exhibiting this protein expression pattern had an open apex morphology while the remaining 2 had a closed apex morphology; 3 of the 4 patients were white; and 3 of the 4 patients were female (**Table 78; Table 83**). There was no direct correlation between the 3 white and the 3 female patients.

5.4.7 The Effect of an Extended Recovery Period on Protein Expression

In order to determine if an extended recovery period of 16 hr, in ambient oxygen supplemented with 5 % CO₂, had any effect on the protein expression profile of human

dental pulp explants exposed to chronic hypoxia (n = 4) compared to that of untreated controls (n = 4), a qualitative assessment was performed on data produced by RayBiotech, Inc. It was shown that for most of the samples, analyte concentrations were calculated to be 0 pg/ml. FGF2 was the only protein of interest in which all samples (n = 8) produced analyte concentrations greater than the assay's lower LOD (**Table 84**). There were no strong trends ($\geq 80\%$) observed in protein expression between the control state and chronic hypoxic exposure in pulps allowed to recover for 16 hr in supplemented ambient oxygen, with the exception of FGF2 which was increased in 3 of the 4 patients studied (**Table 84**). As no strong trends were observed among either the patients or the angiogenesis-specific proteins, phenotypic factors could not be considered as a cause for protein expression patterns.

Data from the extended recovery was then compared to samples treated with chronic hypoxia exposure and snap-frozen immediately after the 30 minute equilibration (**Table 83**). A summary table of this comparison and the overall trends in expression are given as **Table 85**. It was shown that following a prolonged incubation, the protein expression patterns were different to those of samples snap-frozen directly after exposure. TEK protein was decreased in abundance in both the immediately frozen and the prolonged incubation sample groups.

GENES OF INTEREST	LIMIT OF DETECTION pg/ml	PATIENT 1		PATIENT 2		PATIENT 3		PATIENT 4	
		CONTROL	CHRONIC	CONTROL	CHRONIC	CONTROL	CHRONIC	CONTROL	CHRONIC
ANGPT1	105.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FGF2	260.2	1826.0	1898.1	2035.7	2729.8	1628.2	2812.2	1678.3	319.2
KDR	19.5	0.0	0.0	0.0	3.4	0.0	0.0	0.0	0.0
MMP2	301.9	0.0	0.0	0.0	139.6	0.0	634.4	0.0	0.0
PDGFA	23.8	0.5	0.0	13.9	14.2	0.0	0.0	9.2	9.2
PDGFB	2.9	0.0	0.0	0.0	1.8	0.0	0.0	0.0	0.0
SELE	67.2	73.1	29.1	33.9	21.8	0.0	16.5	41.2	57.8
TEK	64.6	0.0	28.8	0.0	0.0	0.0	0.0	31.1	10.3
TGFB1	444.0	0.0	19.1	0.0	0.0	0.0	0.0	0.0	25.4
VEGFA	7.9	1.9	0.0	0.4	0.0	0.0	0.0	0.0	0.0

Table 84 Inter- and intra-patient trends in protein expression following exposure to chronic hypoxia and 16 hr recovery in supplemented ambient oxygen

Cells marked in purple represent samples that expressed a protein concentration greater than the assay's lower LOD. Cells marked in white represent samples which were below the assay's LOD. Cells marked in green represent samples which did not express any detectable concentration of the protein of interest.

SAMPLES SNAP-FROZEN IMMEDIATELY FOLLOWING EXPOSURE						
GENE OF INTEREST	PATIENT 1	PATIENT 2	PATIENT 3	PATIENT 4	PATIENT 5	OVERALL TREND
ANGPT1	-	-	-	-	-	-
FGF2	DOWN	DOWN	DOWN	DOWN	UP	DOWN
KDR	-	-	-	-	-	-
MMP2	-	-	UP	-	-	UP
PDGFA	UP	-	UP	-	-	UP
PDGFB	UP	UP	DOWN	DOWN	DOWN	DOWN
SELE	UP	DOWN	UP	-	UP	UP
TEK	DOWN	-	DOWN	DOWN	-	DOWN
TGFB1	-	-	-	-	-	-
VEGFA	-	-	-	-	-	-
SAMPLES INCUBATED FOR 16 HOURS FOLLOWING EXPOSURE						
GENE OF INTEREST	PATIENT 1	PATIENT 2	PATIENT 3	PATIENT 4		OVERALL TREND
ANGPT1	-	-	-	-		-
FGF2	UP	UP	UP	DOWN		UP
KDR	-	-	-	-		-
MMP2	-	-	-	-		-
PDGFA	-	UP	-	DOWN		-
PDGFB	-	-	-	-		-
SELE	DOWN	DOWN	-	UP		DOWN
TEK	-	-	-	DOWN		DOWN
TGFB1	-	-	-	-		-
VEGFA	-	-	-	-		-

Table 85 Protein Trends following Exposure to Chronic Hypoxia: A Comparison of Snap-freezing Post Exposure and Prolonged Incubation

5.5 Discussion

Angiogenesis or the growth of neovessels from a pre-existing vascular bed is known to be regulated by a number of mechanisms, including hypoxia (Pugh and Ratcliffe 2003). Hypoxia occurs when oxygen concentrations have fallen below the physiological requirements of the cell or tissue, despite an adequate flow of blood to the region (Dorland 2007). It has been a popular topic of investigation in pathological conditions such as tumour metastasis (Semenza 2010), pulmonary hypertension (Bonnet *et al.* 2006) and diabetes linked to obstructive sleep apnoea (Polotsky *et al.* 2003); and is known to occur in a range of intensities from acute to chronic and for varied durations from intermittent to sustained hypoxia. The severity of hypoxia experienced in the cell or tissue has been shown to induce hypoxic activity by distinctly different mechanisms (Yuan *et al.* 2008).

We have previously identified that an orthodontic force, applied to teeth for a period of two weeks prior to extraction, may induce hypoxia in conjunction with a potential angiogenic response within the human dental pulp (**Chapter 4**). The human dental pulp is a highly vascularised tissue (Vongsavan and Matthews 1992, Yoshida and Ohshima 1996) and is a known source of pluripotent stem cells (d'Aquino *et al.* 2009, Pisciotta *et al.* 2015, Tatullo *et al.* 2015); this has brought this tissue to the forefront of many translational research investigations.

To date, no investigations have been performed in order to determine the angiogenic response of human dental pulps to a range of hypoxic intensities or durations. The outcome of such an investigation would potentially enable researchers to more fully comprehend pathological changes observed within the tissue in the fields of reparative, restorative and clinical dentistry; and in stem cell research where pathological interventions outside the field of dentistry are considered.

For this purpose, untreated human dental pulps were isolated from the pulp chambers of healthy premolars and were exposed to intermittent, chronic and repeated cycles of intermittent hypoxia under *in vitro* culture conditions. Transcriptomic and proteomic differences in the pulps were then compared using primers and antibodies, respectively, for a small panel of angiogenesis-specific growth factors, cytokines and chemokines.

5.5.1 Verification of the Intensity of Hypoxic Exposure Experienced by Human Dental Pulps by Measuring Oxygen Tension

In order to verify the intensity of hypoxia at which the human dental pulp explants were exposed, we analysed the oxygen concentrations of the cell culture media in which these tissues were incubated. Analysis was performed using both a Galvanic dissolved oxygen probe and a FOXY probe; with the Galvanic probe being used on media collected from flasks which had contained hypoxically exposed pulp explants and the FOXY probe being used on pulp-free hypoxically exposed media (in order to verify our data). Both probes calculate the oxygen concentration in different manners and it was found that these methods are not corroborative.

The Galvanic probe was zeroed by inserting the sensor into a diluted sodium sulfite (oxygen scavenging) solution and then calibrated by exposing the sensor to the ambient oxygen of the open cell culture cabinet and setting the corresponding reading to 21 % as per manufacturer's instructions. The ambient oxygen concentration, hypothesised to be 21 % by the manufacturer, was considered to be the maximal value of all further readings; and all readings taken from the hypoxic culture media was compared directly against this maximal value.

The FOXY probe calibration was performed by Ms. S. Taylor in the laboratory of Prof. T. Arnett at University College London and oxygen percentage was measured in a

photoluminescence-quenching reaction based on a phase shift from a state of complete anoxia to hypoxia, normoxia or hyperoxia.

When we attempted to align the oxygen concentrations in cell culture media measured with the Galvanic probe to oxygen concentrations measured with the FOXY probe, we found large disparities between the data. We have shown that correlating the Galvanic probe readings to an hypothesised 21 % of ambient oxygen gives vastly different results to those observed by the FOXY probe; and that in order to align the data, we had to assume that the ambient oxygen concentrations for the Galvanic probe were not only much lower than the recommended 21 % but differed greatly according to the exposure of hypoxia. This was both unexpected and unusual but in order to align our data and confirm the intensities of hypoxia to which the pulps were exposed, we created a line of best fit from the FOXY probe data which showed pulp-free cell culture media to have the following oxygen concentrations: (a) control 15.4 %, (b) intermittent hypoxia 6.6 %, (c) repeated cycles of intermittent hypoxia 4.24 %, (d) chronic hypoxia 6.54 %.

Despite creating a line of best fit, based upon the more accurate FOXY probe readings, when our Galvanic probe data was corrected, a number of disparities still existed. We propose that these disparities are due to not only the nature of the Galvanic probe, which requires continual manipulation in the solution in order to quantify an oxygen concentration, but may be attributed to the inability to equilibrate the FOXY media for a period of 30 min after hypoxic exposure due to time limitations of Ms. S. Taylor at University College London.

We confirm that all pulps exposed to any given hypoxic exposure fell within a small range (0 % – 0.5 %) of the other pulps in their treatment group and that all intensities of hypoxic exposure were independent of each other. We suggest that should any further work be performed where oxygen concentrations need to be determined from cell culture media, the FOXY probe should be utilised in place of the Galvanic probe as

readings are not only more accurate (requiring little to no manipulation of the raw data) but is both quicker and easier to use, requiring minimal manipulation of the sample.

5.5.2 Relative Expression of Angiogenesis-Specific mRNA in Pulps Exposed to Hypoxia

Following exposure to varying hypoxic intensities, the explanted pulp halves were homogenised and RNA_{total} was isolated for the purpose of RT-qPCR. Data was corrected for biological variability within the samples, as this was previously established to be a fundamental technique for the analysis of this experimental tissue (**Chapter 4**). It was shown that each hypoxic exposure resulted in a unique transcriptomic expression pattern, this confirmed our *a priori* hypothesis and linked to the literature where it has been described that hypoxic activity is induced by different mechanisms in cells exposed to chronic hypoxia as opposed to intermittent hypoxia (Yuan *et al.* 2008).

5.5.2.1 Intermittent Hypoxia Group

After exposing pulps to intermittent hypoxia and comparing the expression of angiogenesis-specific growth factor mRNA, relative to control explants which had not been exposed to the hypoxic gas mix, we identified a statistically significant increase in the expression of ANGPT1, MMP2, ROBO4, TGFB1 and TGFBR1. We also observed a statistically significant decrease in IL8 and ITGB3 mRNA. In addition to this we observed a decrease in the expression of HIF1A and NOS2 and an increase in PDGFB, although these differences did not reach a level of statistical significance.

Using STRING (www.string-db.org), an online database of predicted protein-protein interactions, and KEGG (<http://www.genome.jp/kegg/>), we were able to determine any existing links in the literature between these genes. In the significant down-regulation of IL8 and ITGB3 mRNA expression, there were no determinable links between the genes

by cell signalling pathway or within the literature. ITGB3 is a cell-surface protein which participates in cell adhesion and cell-surface mediated signalling (Saalbach *et al.* 2005, Shattil and Newman 2004); and was not shown to be linked to any of the other GOIs which were down-regulated following exposure to this intensity of hypoxia.

IL8 is a pro-inflammatory chemokine that has previously been shown to be enhanced by NOS2, another pro-inflammatory chemokine (Vuolteenaho *et al.* 2009). In the intermittent hypoxia group, both of these genes were down-regulated, suggesting that the expression of these genes is correlated and that pulps did not become inflamed following short-term exposure to low oxygen concentrations.

The down-regulation of HIF1A expression also suggests that in addition to the absence of inflammation, the tissues are in a normoxic state following treatment. This may be considered to be irregular, as tissues have been exposed to a depleted source of oxygen for a period of time; but can be explained by the reoxygenation stage of the experimental protocol, where tissues are allowed to recover and revert back to a normoxic state. This reoxygenation stage is fundamental in our attempt to mimic the true physiological state of the tissue, as intermittent hypoxia has been defined as the “periodic exposure to hypoxia, interrupted by a return to normoxia or less hypoxic conditions” (Powell and Garcia 2000). Short-term exposure to hypoxia in the absence of reoxygenation might be mistaken for mild chronic hypoxia; therefore, this reoxygenation stage is critical in our replication of the physiological environment of intermittent hypoxia.

By analysing the GOIs shown to be significantly up-regulated following intermittent hypoxia, several combinations of genes were shown to be linked by cell signalling pathways and within the literature; although no signalling pathway or publication linked all up-regulated genes together. ROBO4 was the only gene which was not shown to be

linked to the other up-regulated genes by pathway or within the literature; neither was it shown to be linked in expression to the non-significantly up-regulated gene, PDGFB.

ROBO4 is an endothelial specific protein which plays a role in vascular permeability and the maintenance of vascular integrity (Ishikawa *et al.* 2010, Jones *et al.* 2008). The increase of ROBO4 mRNA suggests that the vasculature within tissues, having experienced intermittent hypoxia, becomes unstable and is prone to leakage. This finding was supported by the significant up-regulation of MMP2, a matrix metalloproteinase enzyme which is responsible for the degradation of extracellular matrix components. MMP2 has previously been shown to play a role in the degradation of endothelial basement membrane following hypoxic exposure (Ben-Yosef *et al.* 2005); and we postulate that the extent of vascular permeability may be regulated by balancing the expression of MMP2 and ROBO4 proteins, although this has yet to be confirmed. Should our findings be attributed to the reoxygenation stage of the experimental protocol, we suggest that MMP2 expression is induced by hypoxia as part of the angiogenic response and that ROBO4 expression is induced during the reoxygenation stage as part of a recovery from hypoxia in order to stabilise the existing vasculature.

According to STRING analysis, MMP2 was shown to be linked to angiogenesis and tumour development in combination with TGFB1 and TGFBR1 expression in the literature (Gilles *et al.* 1996, Landskron *et al.* 2014, Zarzynska 2014); there was no signalling pathway which linked all three genes directly.

TGFB1 is an anti-inflammatory cytokine which is known to inhibit the expression of both IL8 and SELE (Smith *et al.* 1996), this was confirmed in the response of the tissue to intermittent hypoxia. Whilst TGFB1 is known to induce angiogenesis, it is concurrently known to inhibit endothelial cell proliferation, induce endothelial cell death, down-regulate KDR expression (Ferrari *et al.* 2009) and induce the up-regulation

of MMP2 (Kim *et al.* 2007). The concentration of extracellular TGFB1 seems to have a vital role on the activity of this protein in angiogenesis where low concentrations of extracellular TGFB1 appear to promote endothelial cell proliferation and migration, whilst high concentrations lead to cytoostasis and synthesis of extracellular matrix proteins which are associated with mature and stable vessels (ten Dijke and Arthur 2007). It is not evident from the increases in mRNA expression as to whether TGFB1 protein concentrations are low or high in the extracellular space. Nor is it evident whether this gene is acting in collaboration with MMP2 to promote cell migration, or if it is assisting ROBO4, ANGPT1 and PDGFB in stabilising the existing vasculature; this has yet to be investigated at the proteomic level.

We have described TGFB1 as having both a positive and negative effect on angiogenesis, inflammation and cell death; and these effects are dependent on extracellular concentration of this protein. In pulp explants treated with intermittent hypoxia, we observed an increase in the expression of TGFB1 and its receptor TGFBR1. TGFBR1 has been shown to have a negative regulatory effect in combination with TGFB1; where the activation of this cell signalling pathway leads to an inhibition of endothelial cell migration and proliferation (Lebrin *et al.* 2005). We can therefore postulate that following exposure to intermittent hypoxia, TGFB1 binds to TGFBR1 in order to regulate the angiogenic response and inhibit any further vessel growth; and that this gene combination will act in conjunction with that of ROBO4, ANGPT1 and PDGFB. Although, before any conclusive statement can be made, this should be verified at the proteomic level.

In the pulp explants treated with intermittent hypoxia, we observed an increase in the expression of ANGPT1 and PDGFB. ANGPT1 has been shown to play a role in recruiting pericytes to maturing vasculature (Krock *et al.* 2011), as has PDGFB (Nishishita and Lin *et al.* 2004). This concurrent up-regulation of both ANGPT1 and

PDGFB is in contradiction to the literature where a collaborative increase of ANGPT1 and TGFB1 is supposed to suppress the increase in PDGFB expression (Nishishita and Lin 2004). PDGFB has also been shown to be down-regulated by an increase of TEK expression in HUVEC cells but this down-regulation did not occur despite the non-statistically significant increase in TEK expression within these tissues. (Uebelhoer *et al.* 2013). The mechanism by which PDGFB is up-regulated in these samples is not understood but it is postulated that it will differ from those outlined in Nishishita *et al.* and Uebelhoer *et al.*'s publications, as according to Nilsson *et al.* capillary cells will respond to hypoxia by increasing PDGFB mRNA expression (Nilsson *et al.* 2004).

By STRING analysis of the intermittent hypoxia group, we observed that MMP2 was linked to ANGPT1 and TGFB1 expression in the literature (Goel *et al.* 2011, Riabov *et al.* 2014, Taube *et al.* 2012); and a number of signalling pathways linked these genes together as pairs. There was no single signalling pathway which linked all three genes. MMP2 was linked to TGFB1 in the 'Pathways in cancer' and the 'Proteoglycans in cancer' signalling pathways. TGFB1 was linked to ANGPT1 in the 'Rheumatoid arthritis' signalling pathway and MMP2 was linked to ANGPT1 via the 'Control' curated pathway published on the NCI-Nature Pathways Interaction Database (<http://www.ndexbio.org/>). ANGPT1 is required in order to promote stability of the vasculature by recruiting pericytes to the vessels and TGFB1 has been shown to have a positive effect on vessel stabilisation, when expressed in conjunction with its receptor TGFBR1. At this time, we cannot make any conclusive statements as to the interaction of these two genes in the angiogenic response of human dental pulps to intermittent hypoxia, as proteomic analysis needs to be performed.

MMP2 and ANGPT1 were both shown to be linked in the literature (ElAli *et al.* 2014, Neve *et al.* 2014) and were significantly up-regulated in the intermittent hypoxia treatment group. The concurrent increase in both of these genes appears to be

contradictory in that MMP2 is responsible for destabilising the vasculature whilst ANGPT1 is responsible for attracting pericytes, stabilising vessels and ensuring that vessels do not leak. It is proposed that ANGPT1 mRNA expression is increased in the reoxygenation stage of the experimental protocol, however, this requires further investigation.

5.5.2.2 *Repeated Intermittent Hypoxia Group*

Following repeated cycles of exposure to intermittent hypoxia, we observed no statistically significant differences in the mRNA expression of any of the GOIs. This was not expected as repeated cycles of intermittent hypoxia have been shown to have a greater negative effect on the body, as they have been shown to trigger multiple pathologies (Dale *et al.* 2014). For this reason, repeated intermittent hypoxia is often referred to in the literature as ‘chronic intermittent hypoxia’ due to its sustained nature.

Despite the changes not reaching a level of significance, we observed an increase in ANGPT1, HIF1A, ITGAV, MMP2, ROBO4, SELE, TEK, TGFB1, TGFBR1 and VEGFA mRNA. We also observed a decrease in the mRNA expression of FGF2, IL8, ITGB3, KDR, NOS2, PDGFA and PDGFB.

When analysed by STRING, we could find no more than five of the ten up-regulated GOIs to be linked by a single cell signalling pathway. HIF1A, ITGAV, MMP2, TGFB1 and VEGFA were shown to be linked via the ‘Pathways in cancer’ and ‘Proteoglycans in cancer’ signalling pathways. ANGPT1, HIF1A, TEK and VEGFA were shown to be linked via the ‘HIF1 signalling pathway’. ANGPT1, ITGAV, TEK and VEGFA were linked via the ‘PI3K-Akt signalling pathway’. ANGPT1, TEK, TGFB1 and VEGFA were linked via the ‘Rheumatoid arthritis signalling pathway’. ANGPT1, TEK and VEGFA were linked via the ‘Rap1 signalling pathway’ and ‘Ras signalling pathway’. HIF1A, TGFB1 and VEGFA were linked via the ‘Renal cell carcinoma’ pathway.

TGFB1 and TGFBR1 were linked via the curated 'Biochemical Reaction: UCHL5 or USP15 deubiquitinates TGFBR1' and 'Biochemical Reaction: SMURFs/NEDD4L ubiquitinate phosphorylated TGFBR1 and SMAD7' pathways which are published on the NCI-Nature Pathways Interaction Database (<http://www.ndexbio.org/>).

The down-regulated GOIs were also assessed by STRING analysis and a maximum of five of the seven genes were shown to be linked via a single cell signalling pathway. FGF2, IL8, NOS2, PDGFA and PDGFB were shown to be linked via the 'Pathways in cancer' signalling pathway. FGF2, KDR, PDGFA and PDGFB were shown to be linked via the 'Rap1 signalling pathway', 'Ras signalling pathway' and 'PI3K-Akt signalling pathway'. IL8, KDR, PDGFA and PDGFB were linked via the 'Cytokine-cytokine receptor interaction' pathway. FGF2, PDGFA and PDGFB were linked via the 'Melanoma', 'Regulation of actin cytoskeleton' and 'MAPK signalling pathway'. KDR was linked to PDGFA and PDGFB via the 'Focal adhesion' pathway. IL8 was linked to NOS2 via the 'Amoebiasis' pathway.

We postulate that the increase in VEGFA, a mitogenic factor specific to endothelial cells, has been induced by the increase in HIF1A expression; and that in spite of the reoxygenation stage of the experimental protocol these cells are in a sustained state of cellular hypoxia. VEGFA is known to be hypoxia-inducible and the mRNA expression of this gene is moderated quickly in order to aid in the physiological response to low oxygen concentrations (Brogi *et al.* 1994, Pugh and Ratcliffe 2003, Shweiki *et al.* 1992). As VEGFA expression levels will ordinarily return to normal following reoxygenation of the tissue, the finding that VEGFA mRNA remains up-regulated supports the statement that these pulps remain in a sustained state of hypoxia following the final reoxygenation stage of the experimental protocol.

It is interesting to note that should these tissues be in a sustained state of cellular hypoxia, there appears to be no corresponding increase in inflammation in these tissues.

This is evident from the decrease in IL8 and NOS2, which are both pro-inflammatory chemokines; and from the decrease in FGF2 expression which is also known to be associated with inflammation (Andres *et al.* 2009, Presta *et al.* 2009).

The absence of inflammation appears to contradict the finding that SELE is increased in these explants. SELE protein is synthesised when vascular endothelial cells are stimulated by pro-inflammatory cytokines (Kawase *et al.* 2009), in order to attract, bind and activate circulating leukocytes to the site of inflammation or injury (Kraiss *et al.* 2003, Winkler *et al.* 2012). We propose that inflammation does occur within these tissues during the repetitive cycles of hypoxic exposure in order to up-regulate the expression of SELE; however, we postulate that the reoxygenation stage is sufficient to induce a down-regulation of these GOIs. The mechanisms surrounding this hypothesised response needs to be verified in future work.

FGF2 is a pleiotropic factor which acts in a paracrine, intracrine and autocrine manner (Delrieu 2000, Gomm *et al.* 1997, Kim and Ma 2013, Lefevre *et al.* 2009, Presta *et al.* 2009, Stachowiak *et al.* 1997); and is known to be associated with inflammation and angiogenesis (Presta *et al.* 2005, Presta *et al.* 2009). With respect to angiogenesis, FGF2 is known to regulate the expression of extracellular matrix components, induce neovascularisation, stabilise vasculature and recruit pericytes through the induced expression of PDGFB (Andres *et al.* 2009, Presta *et al.* 2005, Presta *et al.* 2009). In pulp explants exposed to repeated cycles of intermittent hypoxia, we have observed a decrease in FGF2 and PDGFB expression. These findings are contrary to what was expected, as the induction of angiogenesis requires both the regulation of extracellular matrix components and stabilisation of neovessels.

The decrease in PDGFB expression was accompanied by increased expression of ANGPT1, TGFB1 and TEK, suggesting that the recruitment of pericytes to vessels is being suppressed by homeostasis and that neovessel creation is occurring (Nishishita

and Lin 2004, Uebelhoer *et al.* 2013); however, the homeostatic regulation of PDGFB by ANGPT1, an alternate pericyte recruiting agent, seems contradictory. It has previously been observed that an increase in ANGPT1 expression can accompany a decrease in PDGFB expression in the pathology of tissue with polycystic ovary syndrome, where abnormalities in angiogenesis are reported to occur (Di Pietro *et al.* 2015). Whether tissues being exposed to repeated cycles of intermittent hypoxia experience abnormal angiogenesis or whether the expression of PDGFB is regulated by increased ANGPT1 expression remains to be confirmed.

The increase in ANGPT1 expression was supported by an increase in ROBO4 expression, suggesting that during the repeated exposure to intermittent hypoxia, the vascular permeability of existing and possible neovessels is regulated in response to the vasculature becoming leaky and permeated. The incidence of leaky vessels is supported by the increased expression of MMP2 which is required for the degradation of endothelial cell basement membrane components and endothelial cell migration.

In conjunction to the increase in ANGPT1 expression, we observed a corresponding increase in the expression of its receptor, TEK. TEK is an endothelial specific receptor which plays a functional role in the interaction between endothelial cells and smooth muscle cells, such as pericytes (Fukuhara *et al.* 2010, Martin *et al.* 2008). It has previously been shown to be stimulated by hypoxia, although the response to hypoxia was dependent on the source of the endothelial cells examined (Willam *et al.* 2000). This suggests that the responses seen in this tissue may be unique to the pulp and that the mechanisms regulating hypoxia may not only be specific to the intensity of hypoxia (Yuan *et al.* 2008) but may be unique to the complexity of each tissue and its surrounding environment; although this hypothesis has yet to be confirmed.

PDGFA is required for epithelial cell proliferation and has been associated with increased proliferation of mesenchymal stem cells but is primarily active during

embryogenesis (Landskron *et al.* 2014, Mizuno *et al.* 2015, Sukhotnik *et al.* 2012). The down-regulation of the mRNA expression for this GOI was expected in our experimental model.

Unlike VEGFA, its receptor KDR was decreased in pulp tissue explants that had been exposed to repeated cycles of intermittent hypoxia. This was not expected, as an increase in VEGFA should require an increase in receptor for adequate binding and initiation of angiogenesis. KDR mRNA expression has previously been shown to be regulated by hypoxia in an oxygen dose-dependent manner (Takagi *et al.* 1996); and it was postulated by the authors that the initial decrease in KDR may be attributable to tight regulation of the angiogenic response. This can be better described as a lag-period between increased ligand and receptor expression, in order to ensure that an appropriate response is considered and acted upon by the cells. The lag-period allows for cellular oxygen to either return to a state of normoxia or maintain at the hypoxic state and for angiogenesis to be induced accordingly. This lag-period or decrease in KDR expression was not observed by the authors (Takagi *et al.* 1996) in chronic hypoxia and this has yet to be investigated in our study.

TGFB1 has previously been described as having a negative effect on endothelial cell proliferation when it is expressed in conjunction with its receptor TGFBR1. In pulp explants treated with a repeated exposure to cycles of intermittent hypoxia, we observed an increase in both the ligand and the receptor. We can therefore postulate that following repeated exposure to intermittent hypoxia, TGFB1 binds to TGFBR1 in order to regulate the angiogenic response and inhibit any further vessel growth. The duration following inception of intermittent hypoxia, at which each of these genes is up-regulated, has yet to be considered; as TGFB1 may play an initial role in inducing migration and proliferation of endothelial cells in conjunction with another receptor.

5.5.2.3 *Chronic Hypoxia Group*

By exposing pulp explants to chronic hypoxia, there was an up-regulation in the mRNA of all GOIs with the exception of NOS2 and PDGFA, which showed a down-regulation, and PDGFB, which showed no difference between the control and treated pulps. The increase in HIF1A, IL8 and TGFBR1 mRNA expression reached a level of statistical significance.

The down-regulation of PDGFA mRNA expression was expected in this experimental model following the exposure to chronic hypoxia, as PDGFA plays a predominant role in prenatal vasculogenesis and the tissue used in this study was postnatal human dental pulp explants.

NOS2 is an enzyme which produces nitric oxide in order to relax the smooth muscle cells attached to the walls of the vasculature (Aktan 2004, Krock *et al.* 2011); inducing a temporary vasodilation. In contrast to this, NOS2 has also been shown to produce free radicals which act as a cytotoxic agent (Aktan 2004, Jung *et al.* 2000). It was expected that following exposure to chronic hypoxia, there would be an increase in the expression of NOS2 mRNA which would coincide with the vasodilatory actions of this gene; however this was not the case as a down-regulation was determined. In the original study by Toffoli *et al.* (2009), investigators determined that chronic hypoxia induced the repression of genes associated with the inflammatory processes. As NOS2 is considered to be a pro-inflammatory chemokine, we can state that our findings are in accordance with those of the original researchers. The down-regulation of mRNA expression of this gene can also be explained by Zulueta *et al.* (2002) where it was established that hypoxia alone is not sufficient for the induction of NOS2 expression in endothelial cells and that NOS2 can only be induced within an hypoxic state by the pre- and post-transcriptional modification of this gene by alternate cytokines.

The general up-regulation of all other non-significantly different genes confirms our assumption that chronic hypoxia will induce a strong angiogenic response in the human dental pulps. VEGFA and KDR are induced, suggesting that endothelial cell mitogenesis and proliferation is occurring. ANGPT1, TEK and ROBO4 are up-regulated, which suggests that the vasculature is being stabilised following degradation of the extracellular matrix by MMP2 and formation of neovessels; or that leakage of the existing vascular, induced by MMP2 activity, is being regulated homeostatically. ITGAV and ITGB3 are up-regulated concurrently suggesting that they are active in the cell-adhesion of new vasculature. FGF2 and SELE are up-regulated suggesting that inflammation is occurring in the pulps.

The incidence of inflammation was postulated to be suppressed in endothelial cells as a response to chronic hypoxic exposure (Toffoli *et al.* 2009); however, this was not found to be true in human dental pulps where in addition to the up-regulation of FGF2 and SELE, we observed a significant up-regulation of IL8 mRNA expression. This finding negates our previous statement about NOS2 expression and we postulate that in order for NOS2 to be up-regulated in the chronic hypoxic state, this enzyme needs to be modulated by alternate cytokine activity.

STRING analysis was performed on the genes which were found to be significantly up-regulated in response to chronic hypoxic exposure. IL8 was shown to be linked to HIF1A in the literature (Riabov *et al.* 2014, Stockmann *et al.* 2014), whereas TGFBR1 was not shown to be linked to either of the other two genes. There were no signalling pathways that could link any of the genes found to be significantly up-regulated in response to chronic hypoxia.

It is unsurprising that the expression of HIF1A mRNA remains up-regulated following exposure to chronic hypoxia. Prolonged exposure to a limited oxygen concentration is expected to have a greater negative effect than a continual cycle between hypoxia and

reoxygenation. Here we observed a much greater difference in the relative expression of this GOI than we have observed at any of the other treatment intensities. This was also true for the relative expression of the pro-inflammatory cytokine IL8 which had previously been shown to be down-regulated at the other treatment intensities.

The relative expression of TGFBR1 differs in the case of chronic hypoxia as there is no increase in TGFB1 which matches the strength of the receptor's expression, as in intermittent hypoxia. The presence of TGFBR1 without a corresponding expression of TGFB1 is not understood at this time and further investigation is required to determine the function of this gene in the absence of a ligand.

5.5.3 The Effect of Phenotypic Factors and Hypoxic Exposure on mRNA Expression

A qualitative analysis was performed on normalised RT-qPCR data in order to determine trends in mRNA expression within patients and within hypoxic exposure treatment groups. Statistical examination of these trends was not available as the method which corrects for the biological variability of the data requires a calculation of the mean expression of the gene amongst all patients within a treatment group (Willems *et al.* 2008). Established trends were then compared against the patients' phenotypes in order to determine whether a particular phenotypic characteristic would have an effect on the expression patterns of any of the GOIs. It has previously been shown in the literature that the age of the patient is directly linked to the morphology of the tooth apex (Mostafa *et al.* 1991, Ramazanzadeh *et al.* 2009) and therefore the patients' ages were not considered to be an independent phenotypic factor for investigation.

The morphology of the tooth apices has been considered as a significant phenotypic factor, as it has been suggested that pulps with a closed apex morphology will react independently to those with open apices (Hamersky *et al.* 1980, Mostafa *et al.* 1991).

In the intermittent hypoxia treatment group, only a single patient exhibited a strong trend of expression where most of the GOIs were up-regulated following treatment (**Chapter 8, section 8.3.3**). As there were no other patients within this treatment group that exhibited a distinct transcriptomic trend, the phenotypic characteristics of this patient could not be compared or considered as a significant cause of the observed trend. Following repeated exposure to cycles of intermittent hypoxia, we observed a general trend of down-regulated mRNA for the GOIs (**Chapter 8, section 8.3.3**), with the exception of ANGPT1 which was up-regulated in three of the four patients. This result is contrary to that which was observed in the variability corrected relative expression analysis where both up- and down-regulation of mRNA expression was observed. These findings can be explained, as in the qualitative analysis, the baseline expression level of the entire group had not been considered or normalised; and control pulps were paired directly with treated pulps. Amongst this experimental treatment group, three of the five patients were white, three of the five patients were female and three of the five patients had a closed apex morphology. There were no two patients in which all three traits were present and therefore no remark can be made on the effect of phenotype on mRNA expression following repeated exposure of human dental pulps to intermittent hypoxia. Following the exposure of human dental pulps to chronic hypoxia, we observed a general trend towards the down-regulation of mRNA in two of the five patients (**Chapter 8, section 8.3.3**). When phenotypic factors were considered, both pulps were shown to have a closed apex morphology. The relevance of closed apical foramina of the tooth roots has been remarked upon previously, where there is an expected difference in the transcriptomic and proteomic trends of pulps with closed apices in comparison to those with an open apex. Although the pulps had been extracted from their constricting enamel shell for the purpose of these experiments and should have responded in a similar manner to treatment, we propose that histological changes

that occur during tooth maturation and apical closure had altered the cellular composition of the pulp (Andujar *et al.* 1985); and these teeth would behave differently to those with open root apices, whether they were still within the pulp chamber or had been explanted.

When we compared the apex morphologies of all patients within the chronic hypoxia group, three of the five patients were shown to have closed root apices. Therefore, we cannot attribute the changes in mRNA expression to any phenotypic factor within the hypoxia experiments and suggest that in order to truly identify whether phenotypes play a role in gene expression, a larger panel of patients needs to be considered in further investigation.

In an assessment of the effect of each phenotype on the expression of angiogenesis-specific mRNA in hypoxically treated pulps (**Chapter 8, section 8.3.4**), it was shown that patient age had a significant effect on the expression of ANGPT1 and a borderline significant effect on ROBO4. It was previously postulated that transcriptomic differences attributed to a patient's age would be corroborated by the patient's root morphology, as there is a correlation between these two factors. We found that the patient's root morphology had no significant effect on the pulp's response to hypoxic exposure. We could find no other reason for the propensity towards increased mRNA expression of these genes that was related to patient age. Patient gender was shown to have a significant effect on the mRNA expression of MMP2 and TGFB1 although there is no supporting literature to justify or defend these findings. Patient ethnicity was shown to have a significant effect on the expression of ANGPT1, PDGFA and VEGFA; and was shown to have a borderline effect on the expression of FGF2 and TEK mRNA. Whilst there are no publications in the literature that focus on the differences in expression of these specific genes between individual ethnicities, it is now recognised that gene expression levels do differ amongst cell types within an individual, among

individuals and between ethnic groups (Spielman *et al.* 2007). We suggest that further work be carried out on a larger sample group in order to determine the relevance of our findings.

5.5.4 The Effect of Hypoxic Intensity on mRNA Expression

Following a regression analysis on the effect of exposing the human dental pulp explants to any intensity of hypoxia and an examination of the relative differences between each hypoxic exposure, we determined that a number of transcripts are affected by our experimental conditions (**Chapter 8, section 8.3.5**). We determined that exposure of the pulp explants to any intensity of hypoxia would result in a change in the expression of ANGPT1, FGF2, HIF1A, IL8, ITGB3, MMP2, ROBO4, SELE, TGFB1, TGFBR1 and VEGFA. Hypoxia was also shown to have a borderline significant effect on the mRNA expression of ITGAV, KDR and PDGFB. Exposing the pulps to any intensity of hypoxia had no significant effect on the expression of NOS2, PDGFA or TEK mRNA.

As all of the genes which have been shown to change in expression, following exposure to hypoxia, are known to be associated with the angiogenic signalling cascades, we can conclude that exposing human dental pulps to hypoxia will induce an angiogenic response in this tissue. The extent to which the pulp responds will depend on the severity of the hypoxic exposure, as each hypoxic exposure has been shown to result in a unique and distinct transcriptomic reaction.

We have observed in this regression analysis that hypoxia has no effect on the expression of PDGFA mRNA; this was expected as this gene is primarily active in prenatal vasculogenesis and the experimental model that we have investigated is that of postnatal angiogenesis in human dental pulp explants.

TEK mRNA expression was shown not to be correlated with any intensity of hypoxic exposure, this was unexpected as hypoxic exposure was shown to have an effect on the expression of its ligand, ANGPT1. This finding may be due to the small sample of patients which were analysed and we suggest that prior to making conclusive statements regarding the relationship between TEK expression and hypoxic intensity, future work is carried out on a larger group of samples.

NOS2, an inflammatory mediator, was shown to be independent of hypoxia in its mRNA expression. While we hypothesise that inflammation occurs within the tissue in response to hypoxia, we have found no evidence in the literature that claims that NOS2 is regulated by hypoxia. As the sample group that was analysed was a small representative of the general population, we propose that further work be carried out before making a conclusive statement in this regard.

As the results from this analysis did not include the correction for biological variability, unlike the mRNA expression analysis; we propose that the results from the comparisons between the control state and each hypoxic exposure are overlooked and that the data from mRNA expression analysis are considered to be significant for these treatment groups. When assessing the differences between the hypoxic exposures, no correction for biological variability could be considered but these results are of interest as they highlight the potential differences between the mRNA expression levels of the GOIs within two hypoxic intensities.

When comparing the mRNA expression between the individual hypoxic intensities, we observed an increase in the mRNA expression of ANGPT1, IL8, ITGB3, KDR, MMP2, PDFGB, SELE, TGFB1, TGFBR1 and VEGFA in samples treated with repeated cycles of intermittent hypoxia relative to samples exposed to only a single cycle of intermittent hypoxia. In this same treatment group we showed a borderline significant increase in the expression of ROBO4 mRNA.

This finding suggests that following the onset of hypoxia, inflammation is induced in the tissue in response to repeated exposure to intermittent hypoxia. This is evidenced by the increased expression of IL8 mRNA. The increase in KDR and VEGFA suggests that endothelial cells are both migrating and proliferating, this is supported by the increase in MMP2 expression which is required for the degradation of basement membrane molecules in the endothelium. The increase in ANGPT1, PDGFB, TGFB1 and TGFBR1 and the borderline significant increase in ROBO4 mRNA expression contradict these findings as they are associated with vessel stabilisation and the inhibition of endothelial cell proliferation. They may however be attributed to the reoxygenation stage of the experimental protocol or to the homeostatic regulation of vascular permeability.

By comparing intermittent hypoxia to chronic hypoxia, we observed an increase in the mRNA expression of ANGPT1, FGF2, HIF1A, IL8, ITGAV, ITGB3, KDR, MMP2, PDGFB, ROBO4, SELE, TGFB1 and VEGFA.

This finding suggests that following a long exposure to sustained hypoxia, pulp tissues are in a state of oxygen deprivation; this is evidenced by the increased expression of HIF1A mRNA. These tissues are also inflamed as is evidenced by the increase in FGF2, IL8 and SELE mRNA's. The increase in TGFB1 (in the absence of increased TGFBR1), VEGFA and KDR suggests that endothelial cells are both migrating and proliferating, this is supported by the increase in MMP2 expression which is required for the degradation of basement membrane molecules in the endothelium. The increase in ANGPT1, ITGAV, ITGB3, PDGFB and ROBO4 mRNA expression contradict these findings as they are associated with vessel stabilisation and the inhibition of endothelial cell proliferation. They may however be attributed to the reoxygenation stage of the experimental protocol or may be due a homeostatic regulation of vascular permeability.

Between the repeated exposures to intermittent hypoxia and the exposure to chronic hypoxia there was an increase in the expression of ANGPT1, FGF2, HIF1A, IL8, ITGAV, ITGB3, TGFBR1 and VEGFA. There was also a borderline increase in the expression of KDR in this treatment group.

As hypoxia intensifies from repeated cycles of intermittent hypoxia to a more sustained exposure of hypoxia, pulp explants are negatively affected by oxygen deprivation; this is evidenced by the increase in HIF1A mRNA expression. These tissues are also inflamed as is evidenced by the increase in FGF2 and IL8 mRNA's. The increase in VEGFA and KDR suggests that endothelial cells are both migrating and proliferating. The increase in ANGPT1, ITGAV and ITGB3 mRNA expression contradict these findings as they are associated with vessel stabilisation and the inhibition of endothelial cell proliferation. They may however be attributed to the reoxygenation stage of the experimental protocol or may be due a homeostatic regulation of vascular permeability. The increased expression of TGFBR1 appears to be a residual characteristic of the repeated intermittent hypoxic exposure.

5.5.5 Protein Expression Profiles as a Response to Hypoxia

In order to minimise the addition of biological variability to the sample data and to ensure that transcriptomic and proteomic data was directly correlated, four teeth from the same dental position were extracted from a single patient in order to perform our experiments; we used pulps from 5 randomly selected patients. Pulp samples were halved along the mid-sagittal plane and exposed to various intensities of hypoxia. Pulp halves were homogenised and proteins were isolated for analysis by a custom multiplex ELISA. These samples were sent for analysis alongside the orthodontically treated samples from **Chapter 4**. Prior to their delivery to RayBiotech Inc., Georgia, USA, samples were standardised at a concentration of 2 µg/µl following analysis with a BCA

assay. It was identified that in all patients under all experimental treatment conditions, FGF2 was the only protein of interest expressed by the pulps at an analyte concentration greater than the assay's lower LOD. We have previously established in **Chapter 4** that a number of samples returned a concentration of 0 pg/ml for the analytes of interest; however, the number of samples that returned a negative result was greater in the hypoxic exposure experiments. We propose that this was due to the use of half pulps in the hypoxic exposure experiments in comparison to the use of a full pulp explant in the orthodontically treated experiments. Despite the sample homogenates being standardised to a working concentration of 2 µg/µl of total protein, the distribution of the proteins of interest within the homogenates and the initial volume of each protein of interest present in the pulp halves would have a significant effect on the outcome of the ELISA analysis.

The large number of null results may additionally be attributed to the poor specificity of this assay technique. We have previously commented in **Chapter 4** that the use of ELISA in this tissue type is inappropriate as, whilst the tissues appear to have high concentrations of total proteins, there appears to be a very low abundance of the proteins of interest in the samples. We propose, as we have previously, that a high specificity assay that can be used in conjunction with a statistical correction for biological variability is used in future analyses.

We also noted that homogenisation of the pulps and pulp halves with an Ultra-Turrax® homogeniser resulted in some loss of the tissue within the shearing mechanism. Although this had no effect on the analyte concentration of the proteins of interest or the sensitivity of the ELISA assay, we propose at this time that should future investigators wish to homogenise the human dental pulp sample effectively, with minimal sample loss, that an homogenisation technique such as the FastPrep®-24 Instrument or a glass

Dounce tissue homogeniser (previously identified within **Chapter 3**) be used in place of the Ultra-Turrax® homogeniser.

Despite the large number of samples which returned a null or negative result in the ELISA analysis, a qualitative assessment was performed of the data. Analyte concentrations for the proteins of interest were compared between the three hypoxic intensities relative to the untreated controls. Abundances were then assessed between each treatment group to assess the inter-treatment trends. Where one of the samples in the comparison gave a null result, the data pair was excluded from the analysis.

5.5.5.1 Intermittent Hypoxia

We identified that following an exposure to intermittent hypoxia, there was an increased abundance of MMP2, PDGFA, PDGFB, SELE and TEK relative to the control samples. FGF2 was shown to decrease in samples as a response to being exposed to intermittent hypoxia.

Although we have stated that MMP2 increases in abundance in response to intermittent hypoxia, there was only one sample that returned a result in both the control and treated samples. It should also be noted that only one of these values was above the assay's lower LOD and can be considered a true result. MMP2 was previously established to be increased at the transcriptomic level and is thought to play a role in the angiogenic response of human dental pulps to intermittent hypoxia, where vascular permeability and migration is induced by degradation of the extracellular matrix of the existing endothelium. All other increases and decreases in protein abundance at this treatment intensity were not correlated to a significant change in the transcriptome. The only genes which were shown to be expressed in a similar pattern between the transcriptome and the proteome were FGF2, which was decreased in abundance in three of the five

patients investigated; and PDGFB, which increased in abundance in three of the four patients that returned a positive result.

PDGF is a dimeric glycoprotein that can be expressed as either of the homodimers PDGF-AA or PDGF-BB; or as the heterodimeric PDGF-AB (Kaetzel 2003, Meyer-Ingold and Eichner 1995, Shih and Holland 2006). In our ELISA analysis, we attempted to quantify the analyte concentrations of the homodimers, PDGF-AA and PDGF-BB, while PDGF-AB was not considered due to unavailability from the supplier.

We observed an increase in the abundance of both PDGFA and PDGFB in three of the four patients which returned a positive result, although only one patient from the PDGFB group returned both a control and treated concentration value greater than the assay's lower LOD and can be considered a true result.

PDGFA is required for epithelial cell proliferation and has been associated with increased proliferation of mesenchymal stem cells but is primarily active during embryogenesis (Landskron *et al.* 2014, Mizuno *et al.* 2015, Sukhotnik 2012); therefore it was surprising to identify an increase in this protein abundance amongst our samples.

It was even more surprising to identify a synergistic increase in the abundance of PDGFB, as the PDGFA and PDGFB protein chains are subject to different regulatory mechanisms and are produced by different cell types in differing ratios (Dirks and Bloemers 1995, Hoch and Soriano 2003, Kaetzel 2003, Meyer-Ingold and Eichner 1995, Shih and Holland 2006).

PDGFB plays a significant role in angiogenesis, particularly in stabilisation of the neovasculature by pericyte recruitment (Abramsson *et al.* 2003, Andrae *et al.* 2008, Gianni-Barrera 2014, Lindblom *et al.* 2003, Wang *et al.* 2012); and it was expected that there would be an increase in the abundance of this protein following exposure to intermittent hypoxia, as we have already observed an increase in the mRNA expression of this gene at this treatment intensity.

It was expected that an increase in PDGFB abundance would result from an increase in FGF2 expression (Presta *et al.* 2009); however, at this treatment intensity we observed a decrease in FGF2. We therefore suggest that PDGFB is induced by an alternate cytokine, although which cytokine this is has yet to be determined as none of the other proteins of interest which were increased in abundance are known to have any effect on the regulation of PDGFB.

SELE protein is synthesised when vascular endothelial cells are stimulated by pro-inflammatory cytokines (Kawase *et al.* 2009), in order to attract, bind and activate circulating leukocytes to the site of inflammation or injury (Kraiss *et al.* 2003, Winkler *et al.* 2012). The observed increase in SELE abundance, in three of the four patients which returned a positive result, suggests that tissues exposed to intermittent hypoxia are in an inflammatory state during this exposure. This finding cannot be confirmed or denied as there was shown to be no significant increase in pro-inflammatory chemokine mRNA expression and pro-inflammatory chemokines were not assessed within the protein analysis. We propose that in order to substantiate our finding, further work is performed to investigate the proteomic response to hypoxia using specific techniques to identify inflammation.

The abundance of TEK was shown to be increased in two of the three samples which returned a positive result in the protein analysis. This was surprising as an increase in TEK was expected to mediate a decrease in SELE abundance by an anti-inflammatory response (Huang *et al.* 2010). The mechanism by which SELE and TEK protein abundance was increased, in the absences of an increase in pro-inflammatory cytokines, is not understood at this time.

5.5.5.2 *Repeated Intermittent Hypoxia*

When samples were exposed repeatedly to intermittent hypoxia, FGF2 protein abundances were increased whilst PDGFB and TEK was decreased.

This observation was surprising as we had previously determined that there were no significant differences in the mRNA expression in any of the GOIs in treated samples compared to untreated controls. The only gene which exhibited a similar trend in both transcriptomic and proteomic expression amongst these three genes was PDGFB.

PDGFB was found to be increased in mRNA expression and in the protein abundance of two patients which both returned a positive result. Of the patients which returned a positive result in both the control and treated pulp explants, only one of the four analyte concentrations was above the assay's lower LOD. As stated previously, PDGFB is known to play a role in the recruitment of pericytes in order to stabilise the existing vasculature. The increased abundance of PDGFB was expected as during repeated cycles of intermittent hypoxia, we anticipate that a degradation of the extracellular matrix will occur to enable endothelial cells to migrate and proliferate. The degraded, permeated vessels will then require stabilisation in order to ensure they will not leak into the surrounding tissue.

TEK abundance was shown to be increased following repeated exposures to intermittent hypoxia, however it should be noted that only one patient in all five patients evaluated returned a positive result in both the control and treated samples; and neither of these positive results were above the assay's lower LOD.

FGF2 abundance was shown to be increased in four of the five patients that returned a positive result in both the control and treated samples. The increased abundance of FGF2 was not expected as we had previously observed a decrease in the mRNA expression of this gene. We have previously shown that there is minimal post-transcriptional and post-translation modification of this gene (Moffett 1998); therefore,

this finding is irregular and should be evaluated further in a larger sample group. However, as FGF2 is associated with inflammation and SELE mRNA expression was shown to be up-regulated in explants exposed to repeated cycles of intermittent hypoxia, this may be a true observation. Further work is required to validate these findings and to correlate these changes with those from the transcriptome.

5.5.5.3 *Chronic Hypoxia*

Following an exposure to chronic hypoxia, we observed an increased abundance of MMP2, PDGFA and SELE and a decreased abundance of FGF2, PDGFB and TEK.

Previously we had observed the significant up-regulation of three genes at the transcriptomic level. Of these three genes, none were assessed for proteomic expression due to the limitations of the assay. When the proteins which were shown to change in abundance, following exposure to chronic hypoxia, were compared against their mRNA expression trends, we observed an increase in MMP2 and SELE at both the transcriptomic and proteomic levels.

It should be noted that the change in protein abundance observed for MMP2 was only present in one of the five patients analysed, as only this single patient returned a positive result for both the control and treated sample. Of these two positive values which were returned, only one of these values was greater than the assay's lower LOD. The validity of this finding has therefore yet to be confirmed in further investigation. We expect this observation to be true as chronic hypoxia has been shown to be linked to a strong angiogenic response at the transcriptomic level and this includes the degradation of the extracellular membrane for the proliferation and migration of endothelial cells, which is mediated by MMP2.

The increase of SELE protein abundance was shown to occur in three of the four samples that returned a positive result. Of these four samples, only one patient had an

analyte concentration in both the control and treated samples which was above the assay's lower LOD. The remaining three patients each returned a single analyte concentration which was greater than the assay's lower LOD, suggesting that prior to making any conclusive remarks, future work is required to validate our findings. SELE has been shown to be stimulated in response to inflammatory conditions; and we previously observed the increase of FGF2 and IL8 in the chronic hypoxia explants. We propose that this finding is most likely to be true, although future work which considers the proteomic response to inflammation will be required in order to substantiate our hypothesis.

PDGFA which is generally expressed in prenatal vasculogenesis was shown to be increased in both of the two patients which returned a positive result. This was unexpected as the experimental model was postnatal human dental pulps undergoing hypoxia and an anticipated angiogenic response. This is the second observation of this protein following the exposure of pulp explants to hypoxia and we propose that future work is carried out in order to determine the validity of this result. The increased abundance of a prenatal protein in a postnatal response to hypoxia is of interest as it has not been observed prior to this investigation.

FGF2 abundance was shown to be decreased in four of the five patients which returned a positive result following exposure of the pulps to chronic hypoxia. As stated previously, it was expected that protein abundance would change in accordance with the mRNA expression trend, as we have previously identified that there is minimal post-transcriptional and post-translational modification of this gene. Therefore, we propose that further work is carried out on a larger group of samples in order to determine the true changes in FGF2 mRNA and protein following exposure of human dental pulps to chronic hypoxia.

As PDGFB is known to stabilise the vasculature by recruiting pericytes to the walls of the vessels; and we had previously been unable to determine whether there was an increase or decrease in the mRNA expression of this gene, we postulate that our finding is accurate. During chronic hypoxia, there is a sustained low concentration of oxygen; and correcting for this lack of oxygen by establishing neovessels may be a lengthy process. Therefore the stabilisation of the neovasculature by an increase in PDGFB to recruit pericytes could occur at a later duration. We propose that in order to determine whether or not our observation is true, pulp explants are exposed to a longer duration of chronic hypoxia in a strict time-course and proteomic assessment for PDGFB is carried out until an increase in PDGFB abundance is determined.

TEK was shown to decrease in abundance in all three of the patients which returned a positive result in both control and treated explants. TEK is an endothelial cell specific receptor molecule which is involved in the communication between endothelial cells and smooth muscle cells. Like PDGFB, ANGPT1, the ligand of TEK, is responsible for the recruitment of pericytes in order to stabilise vasculature and inhibit vascular leakage. We have already hypothesised that during chronic hypoxia, the need for vascular stabilisation is potentially delayed. We propose that like PDGFB, further work is carried out in order to determine the time-point at which this analyte concentration will increase.

5.5.5.4 Inter-treatment Trends of Protein Expression

Between each hypoxic exposure, we observed a change in the abundance of a number of the proteins of interest. This difference in protein abundance did not match the trends observed in the mRNA expression but did show a distinct/unique difference in protein expression between each treatment intensity. This was expected as the original study by Toffoli *et al.* (2009) outlined that endothelial cells will respond in a different manner

according to the intensity of hypoxia to which they are exposed. This finding was also supported in the literature by Yuan *et al.* (2008), who have stated that hypoxic activity is induced by different mechanisms in cells exposed to chronic hypoxia as opposed to intermittent hypoxia.

Between intermittent hypoxic exposure and repeated cycles of exposure to intermittent hypoxia, we determined that there was an increase in the protein abundances of FGF2 and SELE, with a decrease in PDGFB abundance. In the comparison of expression of mRNA between these two treatment exposures, we also observed an increase in SELE expression. This indicates that exposing the tissue to repeated cycles of intermittent hypoxia will induce an inflammatory state. This hypothesis is evidenced by the concurrent increase in FGF2 analyte concentration but has yet to be confirmed in a larger sample group. The decrease in PDGFB analyte concentration suggests that by exposing pulps to repeated cycles of intermittent hypoxia, there is less requirement for the recruitment of pericytes as vasculature is stable. This observation appears to be contradictory as exposing cells to repeated exposure has been shown to have a more negative effect on samples when compared to the control state. The mechanisms by which angiogenesis is regulated following repeated exposures to intermittent hypoxia needs to be investigated further in order to make any conclusive statements.

When we compared the proteomic expression of pulps exposed to chronic hypoxia to the proteomic expression of pulps exposed to intermittent hypoxia; we observed an increase in KDR, PDGFB and TEK abundances. We also observed a decrease in the expression of FGF2, MMP2, PDGFA, SELE and VEGFA.

The decreased abundances of proteins were not expected in response to a longer duration of hypoxic exposure. These findings appear to be completely contradictory to those discussed previously where an extended exposure has been shown to intensify the angiogenic response of the human dental pulp explants. We hypothesise that the

decreases in protein abundances are not true results but are rather due to a qualitative comparison of very few samples in which most values were below the assay's lower LOD. We suggest that these findings not be considered and that further work is carried out on a larger sample group using a technique that is specific for the detection of low-abundance proteins.

Between pulps exposed to repeated cycles of intermittent exposure and those pulps exposed to chronic hypoxia, we observed an increase in the abundance of KDR and SELE, with a decreased abundance of FGF2, MMP2 and PDGFB. As stated previously, we had not anticipated observing a decrease in the abundance of proteins exposed to a longer duration of treatment. The decrease in abundance of proteins associated with inflammation, basement membrane degradation and vascular stability appears to be contrary to those observations already discussed where tissues move to a more intense state of distress. We propose that these findings are only reflective of the qualitative assessment of several genes where the majority of results returned were null results. We suggest that prior to making any conclusive remarks, further work is carried out using a larger sample group and a technique which is more specific for the detection of low abundance proteins.

5.5.6 Linking Patient Phenotypes to Protein Expression Profiles

We went on from comparing differences in protein abundances between the hypoxic exposures, to identifying trends within a single experimental group. We then examined these trends in order to determine whether or not the phenotypic traits of the patients had any effect on the expression of the proteins of interest.

We could not identify any distinct trends in the expression of any of the proteins of interest within the intermittent hypoxia treatment group and could not therefore attribute any changes in proteomic expression to a single phenotypic characteristic.

When we analysed the protein abundances within the samples exposed to repeated cycles of intermittent hypoxia, we noted a general trend towards increased FGF2 expression in four of the five patients. By investigating the phenotypes of these four patients we noted that two patients were white and the remaining two were black; whilst three were female and the remaining patient was male. Therefore neither patient ethnicity nor gender could be considered as having an effect on the protein expression of the GOIs in this sample group. Upon further analysis of the phenotypes of these patients, we identified that three of these four patients had a closed apex morphology. We have previously discussed our expectations of alternate or differing expression patterns between pulps with a closed apex morphology and those with an open apex morphology. Although three of the five patients that we examined had closed root apices, the remaining two patients did not behave equally to each other in their expected opposing response to treatment. Our sample size was very limited and in order to make a conclusive statement as to the true effect of apex morphology on the pulp's reaction to a series of hypoxic intensities, a much larger sample set is required for further investigation.

In the chronic hypoxia group we established a trend towards the decrease in FGF2 protein abundance in four of the five patients. We observed that two of the four patients had an open apex morphology while the remaining two had closed root apices. Three of the four patients were white and three of the four patients were female. Due to the limitation of sample size, we are hesitant to make any comment as to the effect of ethnicity or gender on protein expression as a response to hypoxic exposure at this point.

As per our study of the mRNA expression trends, we also analysed the effect of patient phenotypes on each of the proteins of interest and details of the full analyses were provided in **Chapter 8, section 8.3.6** and **section 8.3.7**. We identified that in patients

with a closed apex morphology, there was an increase in FGF2 expression in both the ‘control vs intermittent hypoxia’ group and the ‘intermittent hypoxia vs repeated intermittent hypoxia group’. This gene was decreased in expression in patients with an open apex morphology in the ‘control vs chronic hypoxia’ group, the ‘intermittent hypoxia vs repeated intermittent hypoxia’ group and the ‘intermittent hypoxia vs chronic hypoxia’ group. The only treatment group in which protein expression followed the expected hypothesis, of open apex morphology having an opposing trend to that of teeth with a closed apex morphology, was the ‘intermittent hypoxia vs repeated intermittent hypoxia’ group. Patient gender and ethnicity were shown to have neither a corroborative nor opposing expression trend for any of the GOIs at specific hypoxic exposures. As stated previously, we determined that apex morphology is the only phenotypic characteristic that may play a significant role in the pulp’s reaction to hypoxic exposure but in order to validate this finding, further work needs to be considered where a significantly larger panel of patients is considered.

5.5.7 The Effect of an Extended Recovery Period of Protein Expression

In order to determine the effects of a prolonged recovery period from exposure to chronic hypoxia, as per Toffoli *et al.* (2009), we compared the protein expression patterns of pulps that had been exposed to chronic hypoxia directly before snap-freezing to those of pulps which had been allowed to recover from hypoxic exposure for a period of 16 hr. Upon analysis with a custom multiplex ELISA, we determined that many of the GOIs gave an analyte concentration of 0 pg/ml. As discussed previously, this could be attributed to the use of half of the pulp explant in place of a complete pulp; or could be attributed to the level of sensitivity of the assay itself.

FGF2 protein abundance was found to be increased in three of the four patients that had been allowed to recover for the 16 hr period, when compared to those pulps which were

snap-frozen directly after exposure. In the pulps which were snap-frozen directly after treatment, we observed a decrease in FGF2 abundance in four of the five patients examined. It should be noted that any comments on these trends should not be considered as conclusive, as the baseline level of expression in the control samples amongst all nine patients had not been determined. There were no other strong trends in the expression of any of the other proteins of interest but this was due to the large number of null results from the ELISA.

By looking at the general trends of expression in each of the experimental groups, we could determine that the prolonged recovery period resulted in a different protein expression profile overall in all patients. In order to determine if this trend is a true result, we propose that a larger selection of patients be examined in each of the treatment groups and that full explants are analysed using a much more sensitive technique such as FACTT (Zhang *et al.* 2006).

We also propose that should future work be performed in order to determine the effect of prolonged recovery on the proteomic expression profile; the mRNA expression profiles within these samples should also be considered alongside changes in post-transcriptional regulatory factors. We did not perform a comparison of mRNA expression differences in the extended recovery samples as we have previously described that an alteration in cellular mRNA does not always correspond with an increase in protein abundance (**Chapter 4**).

5.5.8 Will Tissue Culture Provide a Representation of the True Physiological Response?

Whilst we have attempted to elucidate the pulp's response to varying intensities of hypoxic exposure, we have done so *ex vivo*; and a number of the regulatory mechanisms that would occur physiologically have been excluded from our experimental model.

Within the hypoxia model, the pulps are no longer encapsulated by dentine, cementum or enamel, nor are they influenced by a homeostatically regulated partial pressure. The pulps have been deprived of a continual supply of nutrients and oxygen, except for that which is present in the culture media; and they have been injured by a mid-sagittal dissection. In the process of flushing the cell culture flasks with the hypoxic gas mix, the pulps within the media are agitated and pulp halves collide against each other and the walls of the cell culture flask, which may induce injury. The pressure of the gas mix during the flushing of the culture flasks may even exfoliate cells from the tissue surface, inducing damage which could alter both the transcriptomic and proteomic responses of the tissue.

We have attempted to minimise the addition of further experimental variability by culturing the pulp halves in medium which had not been supplemented with FCS, a known source of growth factors and cytokines required for cell maintenance and cellular proliferation (Antypas *et al.* 2014, Brunner *et al.* 2010, Gstraunthaler 2003). FCS was not added to our experimental system as any addition of growth factors, cytokines or chemokines could alter the pulp's true response to treatment (Suchanek *et al.* 2013). As we had analysed the protein abundance of a number of these growth factors and cytokines from pulp homogenates, where media on both the tissue surface and within the microvasculature had not been flushed out prior to homogenisation, the exclusion of serum from our experimental model was crucial to determining true responses of the pulp tissue to hypoxic exposure. Pulps were incubated for a short duration of time (up to 16 hr, as per Tofolli's publication) as a more prolonged recovery period may affect the tissue and induce changes that could lead to cellular outgrowth from the explants; not as a response to treatment but as a cell survival mechanism and differentiation of pulp-derived stem cells (Couble *et al.* 2000, Huang *et al.* 2006, Ruch *et al.* 1989, Tawab *et al.* 2009).

It is of import to consider the damage to the tissue by dissection through the mid-sagittal plane. While we gain the benefit of determining both the transcriptomic and proteomic response of a single tissue, isolated from a single patient and thus minimising the biological variability in the sample group; we have induced injury to the tissue and initiated a wound-healing or angiogenic response. Therefore, the changes observed in both the transcriptomic and proteomic analyses may not be solely attributed to the hypoxic response of the tissue.

We propose that further work be carried out in order to optimise the hypoxic model, using whole pulp samples, prior to attempting to determine the true response of pulps to hypoxic exposure. We would recommend that an *in vivo* model is used but appreciate that ethical restrictions may limit this as a possibility. Should this ever become an approved experimental option, careful control and moderation of the cellular oxygen concentrations will be both complicated and problematic.

5.6 Conclusion

In order to investigate the angiogenic response of the human dental pulp to a range of hypoxic intensities, we exposed untreated healthy premolar pulps to a series of intermittent, chronic and repeated cycles of intermittent hypoxia. We have determined in this experimental chapter that:

1. Quantification of oxygen concentrations with a Galvanic probe is an inefficient technique in this experimental model. Not only does this method require manipulation of the sample during testing; but results require multiple stages of correction. In addition to this, data from the Galvanic probe does not correspond with that obtained by spectrophotometric analysis with a FOXY probe. We propose that in all future assessment of oxygen concentration, using this experimental model, that a FOXY probe is utilised.
2. The *a priori* hypothesis that different hypoxic intensities will produce varied and unique transcriptomic and proteomic expression patterns has been confirmed; as each hypoxic intensity appeared to affect the human dental pulp in a distinct manner.
3. The application of any hypoxic intensity to human dental pulp explants will induce an angiogenic response. The severity of the angiogenic response is dependent upon the intensity of the exposure.
4. Transcriptomic analysis of the expression for the angiogenesis-specific GOIs was performed and each hypoxic intensity resulted in a unique expression pattern, relative to normoxic controls. Following exposure to intermittent hypoxia, the human dental explants exhibited a transcriptomic trend towards increased vascular permeability, increased vascular stability, inhibition of vascular proliferation, an absence of inflammation and an absence of hypoxia.

These trends appear to contradict each other but may be attributable to the onset of hypoxia and an angiogenic response, followed by recovery from hypoxia in the reoxygenation stage of the experimental protocol. Following exposure to repeated cycles of intermittent hypoxia we observed a trend towards increased oxygen deprivation, halted or repressed vascular proliferation, vascular stabilisation and an inflammatory response where no pro-inflammatory mediation was evident. We hypothesise that the repeated exposure to hypoxia will induce oxygen deprivation, inflammation and vascular proliferation but that the reoxygenation stages will allow for a small recovery within the tissue. Following exposure to chronic hypoxia, we observed an increase in oxygen deprivation, inflammation, vascular permeability, vascular proliferation and vascular stability. We propose that a prolonged exposure to hypoxia without multiple reoxygenation stages intensifies the negative effects of oxygen deprivation experienced by the pulps; and will concurrently induce a state of inflammation. These tissues will undergo an angiogenic response in order to recover from the negative effects of oxygen deprivation. Vascular permeability is either regulated homeostatically throughout this process or during the reoxygenation stage at the end of the experimental protocol, and is sufficient to allow for a small amount of recovery.

5. It was shown by regression analysis that hypoxic intensity had no effect on the mRNA expression patterns of any particular gene but it did have a notable effect on the expression patterns of genes between two treatment intensities. We hypothesise that following the initial onset of hypoxia the pulp explants will exhibit an induction of inflammation, cell proliferation, cell migration, increased vascular permeability and stabilisation of neovasculature or existing vasculature; should they be exposed to several more hypoxia-reoxygenation cycles. Should

this then intensify to a sustained hypoxia, tissues are hypothesised to experience oxygen deprivation, inflammation, vascular mitogenesis and endothelial cell proliferation in accordance with a strong angiogenic response. Between intermittent hypoxia and sustained chronic hypoxia, we hypothesise that pulps will experience oxygen deprivation, inflammation, vascular mitogenesis and vascular proliferation.

6. The use of an Ultra-Turrax® homogeniser is inappropriate for the homogenisation of human dental pulp explants, especially when samples are halved, as per this experimental protocol. Whilst we were able to recover high concentrations of total protein from our samples, some of the pulp tissue was lost to the homogeniser. We propose that all future work be carried out using a Dounce tissue homogeniser instead.
7. Halving pulp samples had no effect on the mRNA analysis due to the use of the RNA amplification system. By halving pulp samples in the proteomic analysis, we further reduced the total concentration of the low-abundance proteins of interest present in the homogenate. We therefore propose that despite the advantages of minimising biological variability, that halving human dental pulp explants is inappropriate due to the low RNA_{total} and low protein yields of this tissue.
8. The custom multiplex ELISA is an inappropriate technique for the quantification of low abundance proteins in the human dental pulp. We propose that high-specificity techniques, which can be used in conjunction with a statistical protocol for the correction of biological variability, should be used in its place.
9. Following qualitative assessment of proteomic expression patterns, we noted that each hypoxic intensity will result in a distinct pattern. However, this has yet to be verified as a number of samples within the investigation returned a null

value. It was determined that following exposure to intermittent hypoxia protein abundance increased for genes associated with vascular permeability, vessel stabilisation and a response to inflammation. Following repeated exposure to intermittent hypoxia, we observed an increased protein expression trend of inflammation and vascular stabilisation. Following exposure to sustained chronic hypoxia we observed an increased proteomic trend towards genes associated with inflammation, vascular permeability and the expression of PDGFA which is normally active in prenatal vasculogenesis. Between hypoxic intensities we hypothesise that by applying repeated hypoxia-oxygenation cycles to a tissue which has already experienced intermittent hypoxia, there will be an increase in inflammation. We hypothesise that there is an increase in oxygen deprivation, inflammation and vascular proliferation between repeated exposures to intermittent hypoxia and sustained chronic hypoxia.

10. We have established that culturing pulps, previously exposed to chronic hypoxia, under normoxic conditions for a period of up to 16 hr following exposure will result in a distinct and unique protein expression pattern. We hypothesise that a prolonged recovery will have an effect on both mRNA stability, protein translation and protein degradation. The extent to which prolonged recovery affects the pulp explants has yet to be determined as the ELISA analysed only a small number of potential target genes and returned a large number of null results.
11. We have established that patient phenotypes have no significant effect on the mRNA or protein expression of the GOIs in human dental pulps exposed to hypoxia. Apex morphology and patient ethnicity have been highlighted as potential phenotypes which could have an effect on gene expression, although further work is required in order to substantiate this hypothesis.

12. The experimental model used in this investigation removed the pulp tissue from its niche environment where homeostasis is maintained by a number of physiological features. A number of steps within the protocol could agitate the tissue, causing inflammation or wound healing; therefore masking or manipulating true results. We propose that further work be carried out in order to determine a protocol that better replicates the environment of this unique tissue.

In this experimental chapter we have determined that following the exposure to any intensity of hypoxia, an angiogenic response will initiate within the human dental pulp to correct for oxidative deprivation. Each exposure will result in a distinct and unique expression pattern of mRNA and protein which corresponds with the individual hypoxic intensities. The reoxygenation stage of the experimental protocol appears to have a significant effect on the pulp tissue, as a longer duration of hypoxia appears to result in a greater reperfusion injury as oxygen concentrations return to normal.

Chapter VI

General Discussion

The objective of the work within this thesis was to contribute to the current knowledge of the angiogenic response of the human dental pulp to orthodontic force application. This was done with a view to confirming the presence of angiogenesis-specific growth factors, cytokines and chemokines at the transcriptomic and proteomic levels in the tissue and subsequently determining whether key signalling pathways were activated at distinct time-points of routine treatment. Previous studies have identified the presence of several angiogenesis-specific growth factors in cells and tissue explants from human dental pulp; although few have linked these factors to routine orthodontic treatment, thus highlighting the importance of this research.

6.1.1 Experimental Model for Orthodontic Force Application

The experimental model used throughout this thesis was chosen as it would allow for extrapolated data to be linked directly to the physical processes occurring within routine clinical treatment. Patient groups were composed from a mixed ethnical background, a mixed gender population and a narrow age range where tooth morphology was considered to be the most similar. This was performed in order to reduce the introduction of bias and inter-patient variability which could sway the interpretation of data during statistical analyses. Further measures were taken to reduce the introduction of bias or variability at the intra-patient stage by utilising dentally sound contralateral pairs of premolar teeth, sharing similar anatomical attributes. In the experimental chapter attempting to determine the effect of exposure to varying hypoxic intensities, four healthy paired premolar teeth were extracted from each patient from the same dental position. Patients were from a mixed gender and ethnical background and fell within the same age range as the orthodontically treated patients. This was done to ensure that data from all experimental groups could be directly related.

6.1.2 Orthodontic Force Intensity

It is well known that in order to facilitate tooth movement, forces inducing remodelling of the PDL and alveolar bone must be applied without introducing degenerative side effects such as tissue necrosis or hyalinization. While we used what is considered to be a light uninterrupted force (0.5 – 1 N, the equivalent of 50.99 – 101.97 grams-force), it has previously been shown in the rat model that a force greater than 4 grams-force would result in degeneration of the PDL tissue due to the greater level of compression experienced (Noda *et al.* 2009). This has been contradicted in a human study where pulpal blood flow was measured during continuous orthodontic force application and subsequent to force removal; forces within the range of 40 – 50 grams-force were found to be appropriate, as the effects on pulpal blood flow were shown to be fully reversible (Sano *et al.* 2002). Although a number of studies have attempted to identify the ‘optimal force’ where PDL damage does not occur and where pulpal blood flow remains as close to that of an untreated tooth, the aim of our study was to maintain uniformity with that of routine clinical conditions and therefore this level of force (0.5 – 1 N) was selected. Investigators should note that force levels are compared in the literature among a number of experimental models where the physiology of the subject does not always correlate with that of another model. Care should always be taken to try and optimise experimental conditions within the experimental organism of choice.

6.1.3 Optimisation of Experimental Protocols for mRNA Expression Analysis

In the first experimental section we highlighted the necessity for optimisation of thorough working protocols prior to the commencement of gene expression analyses. By trial and error we were able to determine that standard procedures used globally by

investigators in this field could result in detrimental knock-on effects in data production and interpretation.

6.1.3.1 *Sample Storage and RNA Preservation*

We observed that similar RNA_{total} yields can be obtained from samples stored in an RNA preservative compared to those snap-frozen in liquid nitrogen; however, we later learned that an RNA preservative would have a denaturing effect on proteins within the sample. We suggested that for ease of use in a clinical environment, an RNA preservative is used for samples to be analysed by RT-qPCR or microarray; and that snap-freezing be used where tissue needs to be analysed by Western Blot, ELISA or histological processing for protein analysis. The effects of RNA preservatives on a tissue to be processed for *in situ* analysis has not been determined in this study.

6.1.3.2 *Sample Homogenisation*

While a number of homogenisation protocols are used globally for RNA_{total} isolation we found that sample volume was lost during homogenisation in a mortar and pestle in liquid nitrogen; and by passing the sample through a needle in an RNA stabilisation solution. We proposed that the optimal method of pulp homogenisation for independent laboratories, which do not have extensive budgets for equipment and reagents, was to immerse the tissue in an RNA stabilisation solution within a glass Dounce tissue homogeniser and to homogenise the tissue on ice. Should a greater financial budget be available, homogenisation of pulps with the FastPrep®-24 Instrument is recommended.

6.1.3.3 *RNA Isolation Protocols*

Phenol:chloroform RNA isolation was discounted as an appropriate protocol for RNA_{total} isolation from the pulp, as high levels of organic compound contamination were shown to be present at the point of RNA_{total} quantification. The RNEasy Mini Kit

(Qiagen Ltd., UK) without the recommended DNase treatment step was then compared against the phenol:chloroform extraction protocol and found to be notably favourable in comparison. It was suggested that by combining aspects from both the phenol:chloroform and RNEasy Mini Kit RNA isolation protocols, we could increase the yield of RNA_{total} and reduce the level of organic compound contamination. This was shown to be true and we suggest that this method be used in combination with the WT-Ovation™ Pico RNA Amplification System for reverse-transcription as the amplification kit includes a DNA clean-up phase which purifies cDNA and removes potential qPCR inhibitors from the samples.

6.1.3.4 RNA Quantification Protocols

In this section of the study we were able to highlight the large discrepancies between commonly used RNA quantification protocols. By comparing a standard bench-top UV spectrophotometer to the new-age technology of a purpose-built NanoDrop UV spectrophotometer, we were able to note the effects of technological progress on a routine protocol where the physics has not changed but the sensitivity of the assay has. By then comparing NanoDrop UV spectrophotometry to the fluorescence-based assay in the ‘Quant-iT™ Ribogreen® RNA Assay Kit’ we were able to further illustrate the significant differences in the amount of RNA_{total} thought to be obtainable by the UV spectrophotometry method compared with the true mass of RNA_{total} present in a single isolate. While it is noted that at concentrations of 100 ng/μl or more, UV spectrophotometry presents similar yields to the Ribogreen® assay (Bustin 2002), the concentrations obtainable from a single pulp isolate fall well below this level, yielding standard UV spectrophotometry an obsolete protocol in quantification of RNA_{total} isolated from the human dental pulp. It should however be noted that in a survey of nearly 100 researchers from academia, industry and diagnostic laboratories; similar

results were achieved where no correlation was found between quantification methods and it was concluded that it was inadvisable to compare methods, yet prudent to measure all samples within an experiment using the same quantification approach. (Bustin 2005, Nolan *et al.* 2006). Therefore, we do not suggest any preference between analysis with the NanoDrop UV spectrophotometer or the Ribogreen® fluorescence-based assay but have utilised both in this experimental system. We have used the Ribogreen® assay for quantification of our samples and we have used the NanoDrop UV spectrophotometer for verification of sample integrity.

6.1.3.5 Reverse-Transcription Protocols

By identifying the low-RNA_{total} yielding nature of this tissue, we were able to modify our approach to reverse-transcription and subsequently RT-qPCR. We compared a well-known one-step RT-qPCR kit with an RNA amplification system, which in turn resulted in a maximisation of the experimental potential of a single pulp to beyond our original expectations. By utilising the one-step system, we would limit the number of RT-qPCR reactions per pulp to 40 reactions performed in technical duplicate. This was compared against the RNA amplification system which would allow for up to 6,441 RT-qPCR reactions to be performed in technical duplicate, based on an isolated mass of 806.54 ± 33.37 ng RNA_{total} per pulp sample. The identification of the RNA amplification system thus eliminates the need for pooling samples in gene expression studies of the human dental pulp altogether.

It should be noted that the use of a one-step RT-qPCR kit holds the advantage of reducing the potential for introducing contaminants to the reaction. Multiple processes are combined into a single tube and this process does not require as many freeze-thaw cycles of the original sample, should the sample not have been divided into aliquots at the time of extraction (Nolan *et al.* 2006). It has also been noted in the literature that in

two-step RT-qPCR reactions, where $\text{RNA}_{\text{total}}$ is converted to cDNA prior to the amplification reaction; the presence of any residual reverse-transcriptase enzyme may inhibit the amplification assay (Nolan *et al.* 2006). This has been considered in the RNA amplification system as the technique includes a separate phase where the cDNA is desalted and cleaned of any residual enzyme, although the potential for contaminating the sample through many handling stages remains a concern.

6.1.3.6 *Normalisation of RT-qPCR Data: Reference Gene vs. Normalising Factor*

Our attention then turned towards the normalisation of RT-qPCR data. It has been shown in a number of studies that the choice of reference gene or normalising factor (composed from a ratio of reference gene expression levels) will have a knock-on effect on the interpretation of data. This appears to have gone unnoticed amongst researchers in this field who have automatically reverted to the use of popular reference genes and have not considered the stability of other equally important, less prevalent reference genes in their studies. By comparing the expression stability of several reference genes confirmed to be expressed in human dental pulp tissue, and using three recognised and highly respected software packages for expression stability analysis; we were able to determine that in the *in vivo* model of orthodontic force application to human teeth prior to extraction and dental pulp isolation, a normalising factor created from a mean of RPL13A and UBC gene expression was the most appropriate for data normalisation in our experimental system. We also identified that should an experimental group have limited funding, it would be acceptable, although ill-advised, to use the single most stable reference gene UBC as a normaliser. We state that this is ill-advised as we have determined that there will be a number of significant differences in the data obtained by

normalising against a single gene in comparison to a normalising factor; but we leave this decision to the discretion of future investigators.

6.1.3.7 *The Effect of Reaction Efficiency on mRNA Quantification*

In the second experimental section, we identified the effect of amplification efficiency on the under- or over-estimation of mRNA abundances. In the first experimental section, we had identified the statistically significant up-regulation of FGF2 mRNA in pulps which had been treated with orthodontic force application for a period of 2 weeks prior to tooth extraction. When we attempted to verify this result, using a different set of primers, we did not establish the same conclusion. Primers were then assessed for differences between transcript length and inclusion of splice variants; it was established that primers from Primer Design Ltd., UK did not span the same region of the transcript as the primers designed in collaboration with Dr Nick Silver, and did not include the splice variant FGF2-002.

This was thought to be the cause of the discrepancy until the calibration curves for the initial reactions were re-assessed and the amplification efficiency was established as 1.03.

The REST-2009© software package allows for a maximal input value of 1.00 (representative of 100 % or exact doubling of the template) for reaction efficiency, as it is impossible to do any more than perfectly double the original template in each reaction cycle. Therefore, the discrepancy between results from experimental sections one and two were due to the over-estimation of mRNA abundance for this GOI by improper consideration of the amplification efficiency; and not due to the absence of the amplification of the splice variant FGF2-002.

While the REST-2009© software package incorporates a critical reaction efficiency-correction step, the methodology for biological variability correction, which was

utilised later in the project for statistical analysis, does not. Therefore it is essential that before performing the variability correction step on RT-qPCR data, the reaction efficiencies are established to be as close to 100 % as possible and within a small range of each other in order to ensure that data is directly comparable. Correcting for biological variability was only considered to be appropriate in this thesis as efficiency levels were guaranteed by the primer distributor. Had the efficiencies of the reactions not been guaranteed and directly comparable, any variation in efficiency may add to the over- or underestimation of mRNA expression in our samples.

The variability correction step could not be performed on mRNA expression data in the first experimental section, from reactions where we had designed and optimised the reaction primers ourselves; as calibration curves created for the GOIs showed 3 of the 4 genes having a reaction efficiency greater than 100 %, with the reference gene efficiencies being widely varied but below 100 %. The reaction efficiencies of the reference genes were taken into account when assessing reference gene stability and as no efficiency was ranked over 100 %, the results of reference gene stability analysis remain applicable to our experimental conditions.

It is generally considered that within an RT-qPCR reaction, there is an intensity of fluorescence proportional to the DNA concentration present; and this relationship is linear throughout the exponential phase (Ruijter *et al.* 2013). This assumption allows investigators to calculate reaction efficiencies from a straight line graph where known concentrations are input into the reaction and C_q values are related accordingly. This method of determining the reaction efficiency is called the 'constant efficiency' method and assumes that reaction efficiency is constant throughout the exponential phase of the reaction. Following the exponential phase, reaction efficiency is thought to diminish.

An alternate method for calculating the reaction efficiency is to assume that over the course of the reaction there is a continual decrease in reagents and an accumulation of

product, with an increase in binding competition between the primer and the amplicon. Over each cycle, the efficiency is calculated and an average efficiency is calculated for the entire reaction. This method is called the ‘decrease per cycle’ method (Ruijter *et al.* 2013).

The Corbett Rotor-Gene™ 6000 Application Software, version 1.7 (Build 87) does not utilise the ‘decrease per cycle’ method but instead uses the ‘constant efficiency’ method. It is known that by assuming the reaction efficiency to be 100 % for all GOIs across the entire experiment, without evaluating individual target gene calibration curves, will lead to an over-estimation of the data; but in that light so would the use of the ‘constant efficiency’ model for calculating the efficiencies of individual reactions. Should there be a decrease in efficiency due to binding competition between primers and the amplicon; or perhaps the amplification of spurious binding products; the reaction efficiency would be altered without consideration. This is notably a significant factor for any future researcher in this field to reflect upon at the onset of investigation. The concept regarding the over-estimation of data in RT-qPCR was not considered past the point of having an efficiency value greater than the maximal value for the GOIs in our first experimental section and attempting to correct for that in statistical analyses. Prior to being informed of the ‘decrease per cycle’ theory, we had assumed that all which was required in order to obtain valid and statistically significant data, was to optimise each gene’s primers against a calibration curve created from a cDNA library. For this purpose, we had purchased optimised primers where the reaction efficiency was guaranteed to be 100 % and then determined our relative gene expression between control and treated samples accordingly. The suggestion that this method would still lead to an under- or over-estimation of the mRNA abundance of our samples outlines the continual growth in this experimentally young field and highlights the progress which still needs to be made in order to obtain true and conclusive results.

6.1.3.8 *Correcting for Biological Variability in mRNA Expression Data*

Following our identification that all RT-qPCR reactions should have a similar reaction efficiency value, based on literature available at the time of experimentation; RT-qPCR was performed for all samples using primers for each GOI. Data was expressed as C_q values which were then normalised against the expression of the normalising factor, created from the mean expression of reference gene RPL13A and UBC. Data was input into the REST-2009© software package and we could determine no significant differences in the expression of the GOIs at any of the experimental time-points. This was unexpected as previous work carried out by other groups suggested that there was an increase in the protein abundance of our GOIs in our experimental model and this had to be attributed to an initial up-regulation of mRNA expression. When patient data from the control samples was analysed for variability, we determined that each patient had a varying baseline of expression for each GOI when compared to other patients within the sample group. It has been postulated that this is due to the cellular activity and composition of each tissue.

We had previously attempted to minimise the amount of variability present in the sample group by restricting the ages of patients and therefore the correlated tissue morphology of each tooth but this did not limit the variability sufficiently for accurate analysis of the data to be performed.

A statistical model created for microarray data was identified amongst the literature and was broken down into individual steps, our understanding of the methodology was then sent for correction and clarification by Dr N. A. Donaldson, a statistician. Upon clarification of the process for obtaining statistical significance from the data, we analysed all RT-qPCR data and were able to identify a number of statistically significant changes in mRNA expression in our experimental model.

Biological variability has been considered to be an issue in a number of previous studies (Nixon *et al.* 1997, Ricos *et al.* 2009, Smith *et al.* 1993) but the limitation it places on RT-qPCR data, particularly from human dental explants, has not been considered until now.

We propose that following the correction for amplification efficiency, according to the ‘decrease per cycle’ model; the correction for biological variability is the second most critical step for obtaining a significant and valid interpretation of transcriptomic changes occurring within an experimental system. Therefore, all future analysis of the transcriptomic changes in human dental pulp should utilise the statistical method that has been outlined within this thesis.

6.1.4 Angiogenic Response of the Human Dental Pulp to Orthodontic Force Application

Within the second experimental section of this thesis, after having identified all correct protocols for mRNA expression analysis in our experimental model; we quantified and compared the expression of mRNA and proteins specific to the angiogenic signalling cascades in pulps isolated from orthodontically treated teeth. This was done in order to confirm or reject the hypothesised angiogenic response of the human dental pulp, proposed by Derringer *et al.* (Derringer *et al.* 1996, Derringer and Linden 1998, Derringer and Linden 2003, Derringer and Linden 2004), to three stages of early routine orthodontic treatment.

Following 24 hr of force application we observed a concerted increase and decrease in mRNAs associated with the homeostatic regulation of pericyte recruitment to the vessel walls. This was not supported by a corresponding decrease in protein abundance for PDGFB, as six of the ten readings returned from the ELISA analysis were null results.

Although it is indicated at the transcriptomic level, we cannot conclusively state at this stage that the pulpal vasculature is stable following 24 hr of routine clinical treatment.

In addition to this, we observed an increase in the abundance of FGF2 protein. This complemented the non-significant increase of FGF2 mRNA at this time-point of treatment and is associated with inflammation. The suggested state of inflammation at this stage of treatment is substantiated by the increase in mRNA for IL8, a pro-inflammatory cytokine present in pulpal tissue solely in inflammatory conditions; and the increase in SELE protein abundance, where SELE is synthesised by vascular endothelial cells which have been stimulated by pro-inflammatory cytokines. We also observed a non-statistically significant change in the up-regulation of HIF1A mRNA which accumulates at the transcriptomic level only in conditions of oxidative stress and is associated with cellular hypoxia.

After 3 days of force application, we observed corroborative trends in mRNA expression which suggested that hyperoxia and / or inflammation were present in the pulpal tissues at this stage of treatment. These trends included a significant decrease in the expression of HIF1A mRNA, associated with hyperoxia; and a non-significant increase in IL8 mRNA, indicative of inflammation.

There was shown to be a non-significant decrease in FGF2 mRNA which was matched by a decrease in FGF2 protein abundance. This was not expected as an inflammatory response is known to increase the FGF2 abundance. There was also a decrease in SELE protein abundance which would normally increase under inflammatory conditions.

We hypothesised that SELE is under the control of pro-inflammatory cytokines other than IL8 as we maintained the hypothesis that following 3 days of force application, pulps are in an inflamed state.

PDGFB protein abundance was shown to increase at this time of treatment indicating a need to recruit pericytes to vessels and a potential increase in the interstitial fluid

pressure. Due to the increase of PDGFB protein, we concluded that it was less likely that hyperoxia was occurring; as PDGFB is known to decrease in hyperoxia and we assumed that pulps were responding to the initial inflammatory stimulus at 24 hr of treatment. Given the morphology of the pulp tissue, any increase in the interstitial fluid pressure would prolong the inflammatory state of the organ.

At the 14 day duration of routine clinical treatment there was a significant increase in the expression of HIF1A mRNA, indicating that at this time-point the pulps were once again under oxidative duress. Other genes associated with angiogenesis and coagulation were also shown to be significantly up-regulated at this orthodontic treatment time-point; and this was substantiated by the non-significant increase of pro-inflammatory cytokines IL8 and NOS2.

FGF2 protein abundance increased in accordance with the corresponding non-significant increase of FGF2 mRNA. This increase is thought to be associated with the inflamed state of the pulp. Incidence of inflammation was supported by the increased abundance of SELE protein. The decrease in PDGFB protein at this treatment time-point indicates that either the vasculature is stable or that tissues are attempting to regulate the inflammatory state, where a negative feedback loop is preventing the further increase of interstitial fluid pressure. Although, this requires further investigation in order to make any conclusive statements.

From our findings we postulate that following the initial application of orthodontic force, the pulp experiences inflammation and that this inflammatory response is accompanied by oxidative stress. We propose that the pulpal morphology of mature teeth, with closed apical foramina, maintains the inflammatory state which is then intensified by an increase in interstitial fluid pressure. Although at this point, the initial oxidative stress is relieved. After two weeks of force application, pulps are once again under oxidative stress in conjunction with inflammation and the potential onset of

angiogenesis, however, there is no apparent alteration to pericyte recruitment but there is a potential regulation of interstitial fluid pressure.

At the onset of this experimental section our intent was to verify the findings of Derringer *et al.* (2003, 2004) where angiogenic growth factors were hypothesised to be increased transcriptomically prior to being secreted by the tissue as proteins. We could not confirm or deny all findings of this group but have noted an increase in the transcriptome and proteome of most of these GOIs previously studied by Derringer *et al.* and in some of the GOIs in the extended panel for investigation. At no time in our study did we identify a statistically significant increase in the mRNA expression or protein abundance of VEGFA, one of the leading protagonists in the angiogenic signalling cascades. We suppose that the additional time in which Derringer *et al.* (2003, 2004) continued to co-culture the pulps following isolation, was sufficient to induce both transcriptomic and proteomic up-regulation of this gene; and we hypothesise that this would be in response to hypoxia and inflammation which is evident in our findings following 2 weeks of force application.

Inflammation and hypoxia have been evidenced in the literature as a response of the dental pulp to orthodontic force application and this too substantiates our findings (Romer *et al.* 2014, Yamaguchi 2007). It is also of interest to note that the existing lymph vessels of the pulp tissue are only stimulated to grow under inflammatory conditions and may only be required in order to reduce the interstitial tissue fluid pressure of an inflamed tissue (Gerli *et al.* 2010). It would be of interest for further groups to assess the vasculature of untreated control and orthodontically treated pulps, confirming or denying the presence of hypoxia and inflammation in the tissues; and then to go on and investigate the potential increase of lymph vessels, in order to confirm or deny the initiation of lymph angiogenesis as a potential homeostatic regulator of interstitial fluid pressure in response to orthodontic treatment.

As inflammation has been shown to play a putative role in the response of the human dental pulp to routine clinical treatment, it should be noted that the true inflammatory response of this tissue may not be reflected within our data. During the consultations associated with the initiation orthodontic treatment, patients are made aware of a potential pain response which may result from treatment and were advised to take paracetamol if necessary. It is possible that patients within the one and three day treated groups could have consumed anti-inflammatory medication in order to alleviate pain symptoms experienced as a routine side effect of treatment. These anti-inflammatory medications would have a knock-on effect on the regulation of the transcriptomes and proteomes associated with the inflammatory response in the pulp and periodontal tissues. Patients within the two week treated group are more likely to exhibit true responses, with respect to inflammation; as it is postulated that by this time-point of treatment teeth will be within the lag phase of tooth movement and pain would be minimal. Whilst it is unethical to suggest that patients should refrain from consuming medication to alleviate pain during the initial stages of tooth movement, future researchers would need to take into consideration whether or not anti-inflammatories were consumed and attempt to identify the course of medication adhered to in order to attempt to better understand the role of inflammation in the pulp in response to routine clinical treatment.

6.1.5 The Response of the Human Dental Pulp to Varied Intensities of Hypoxic Exposure

Following on from our determination that during the initial (24 to 48 hr) and lag (up to 20 days after force application) phases of routine orthodontic treatment, pulps experience oxygen deprivation; we attempted to characterise the response of the human dental pulp to varied intensities of hypoxia. We had hypothesised prior to investigation

that defined intensities of hypoxia would yield a response, unique to each corresponding hypoxic intensity; and this was established to be true.

Following an exposure to intermittent hypoxia we observed a transcriptomic trend towards increased vascular permeability, increased vascular stability, inhibition of vascular proliferation, an absence of inflammation and an absence of hypoxia. This was supported in the assessment of protein abundances where we observed an increase in proteins associated with vascular permeability, vessel stabilisation and a response to inflammation. We could not determine corroborative protein abundances for genes associated with the inhibition of vascular proliferation or for genes associated with the inception of inflammation and hypoxia. This was due not only to the number of samples which returned a negative or null result; but was due to the limited number of analytes available in the custom multiplex ELISA. We have postulated that the contradictory protein expression associated with both vascular permeability and vascular stability is either present because of a homeostatic balance of degrading and stabilising mechanisms; or that degradation occurs during the initial hypoxic response and vascular stability occurs during the recovery from the onset of angiogenesis. Homeostatic or potentially reparative mechanisms need to be investigated further in order to make a conclusive statement on the pulp's response to intermittent hypoxia.

Following an exposure to repeated cycles of hypoxia we observed a transcriptomic trend towards increased oxygen deprivation, halted or repressed vascular proliferation, vascular stabilisation and an inflammatory response, where no pro-inflammatory stimulation was evident. In the proteomic analysis we observed an increased protein expression trend of inflammation and vascular stabilisation. We could not determine the proteomic difference in the abundances of other genes associated with hypoxia or inflammation as these analytes were not available for the custom multiplex ELISA.

Following an exposure to chronic hypoxia we observed a transcriptomic trend towards oxygen deprivation, inflammation, vascular permeability, vascular proliferation and vascular stability. We observed a corroborative increase in the proteomic trend towards genes associated with inflammation and vascular permeability, in conjunction with the expression of a prenatal protein specific to vasculogenesis. We were not able to confirm increased expression of genes associated with vascular proliferation, hypoxia or vessel stabilisation due to the number of null results returned and the lack of availability of analytes in the ELISA.

PDGFA, a protein found to be expressed most significantly in the embryo during vasculogenesis and neurogenesis has been shown to be expressed at low levels throughout a number of tissues in the adult, where expression is variable, is regionally restricted and is locally regulated (Andrae *et al.* 2014). It has also been shown to be specifically up-regulated in adult tissues which are mesenchymal-derived (Fecteau *et al.* 2006, Floege *et al.* 2008, Yeh *et al.* 1991) and the presence of its receptor, PDGFRA, has been shown to be a marker of mesenchymal stem cells (Farahani and Xaymardan 2015, Kaetzel 2003). While there has been no evidence to support an increase in the activity of PDGFA in the pulp previously, we hypothesise that this increase in protein abundance is not attributed to differentiated adult cells but rather to active, differentiating mesenchymal-derived human dental pulp stem cells. However, this hypothesis has yet to be confirmed in future work.

The reoxygenation stage of the experimental protocol, within the intermittent and repeated cycles of intermittent hypoxic exposure, has been considered to be a significant factor which will alter the transcriptomic and proteomic expression trends observed within our investigation. Previously, we had postulated that these periods of reoxygenation would allow the tissue a short period of time to recover from the negative effects of oxygen deprivation. We had not considered that the reoxygenation phase of

the hypoxia-reoxygenation or ischemia-reperfusion cycle is associated with reperfusion injury; or that this may be the cause for many of the increased trends which have been observed in the human dental pulp explants.

During ischemia (hypoxia), the intracellular pH decreases, ATP production is impaired and intracellular calcium increases; this causes the cells to swell and cell death is initiated (Kalogeris *et al.* 2012, Saikumar *et al.* 1998.). Cells are rescued from cell death or necrosis by reperfusion, where oxygen concentrations, intracellular pH and consequently ATP production is restored (Eltzschig and Collard 2004, Kalogeris *et al.* 2012). The influx of oxygen results in the enhanced production of reactive oxygen species and these then act as cytotoxic agents to induce oxidative stress (Eltzschig and Collard 2004, Kalogeris *et al.* 2012). The induction of oxidative stress in the endothelium results in leukocyte-endothelial cell adhesion and the initiation of an inflammatory response, which has been shown to lead to coagulation of the microvasculature (Carden and Granger 2000, Granger and Kvietys 2015).

When transcriptomic and proteomic changes in the intermittent hypoxia group were reassessed, the only gene which could be considered to be correlated with reperfusion injury was the increase in the proteomic abundance of SELE. The expression of SELE in conjunction with other cell adhesion molecules is activated by reactive oxygen metabolites in order to sustain leukocyte-endothelial adhesion and maintain the inflammatory response in the tissue (Carden and Granger 2000). As these tissues did not appear to be in an inflammatory state, we cannot conclude that reperfusion injury occurs in the human dental pulp in our defined intensity of intermittent hypoxia.

In our assessment of the pulp's angiogenic response to repeated cycles of intermittent hypoxia, we observed a number of increases in both the transcriptomic and proteomic expression of genes which may correlate with a response to reperfusion/reoxygenation injury. We also observed a number of these changes in our chronic hypoxia group. We

were surprised to note that in the group where the hypoxia-reoxygenation cycles were prevalent, a strong inflammatory response was not induced within the tissues as expected. Inflammation was however observed in the chronic hypoxia group. We therefore propose that the intensity of hypoxia which is experienced in the tissue is directly correlated to the extent of the reperfusion injury which will occur in response to reoxygenation.

By investigating reperfusion injury we had attempted to elucidate the response of the human dental pulp to intermittent hypoxia and the repeated exposure to intermittent hypoxia. Instead, we determined that reperfusion injury is most likely to occur within the chronic hypoxia group under our experimental conditions. In order to more fully comprehend the true angiogenic response of the human dental pulp to chronic hypoxia, where reperfusion injury plays no significant role, we propose that this intensity of hypoxia is reinvestigated in the absence of a reoxygenation stage.

Further to our investigation into the effect of hypoxic intensity on the angiogenic response of the human dental pulp, we investigated the effect of a prolonged recovery period on protein production within the chronic hypoxia group. This was done in an attempt to allow for translation of proteins to occur and was performed in accordance to Toffoli *et al.*'s (2009) manuscript. What had not been considered by the investigators was the continual modification of mRNA and protein expression within the tissues in an attempt to maintain homeostasis. Mechanisms of post-transcriptional and post-translational modification had also been omitted from consideration.

The prolonged recovery period outlined in the experimental protocol was intended to enable the observation of previously undetermined increases in protein abundance following treatment. However, as tissues are likely to attempt a correction, during the recovery period, for the oxygen deprivation experienced during hypoxic exposure; the protein result observed directly after hypoxic exposure can never be directly compared

to those from an extended recovery period. It should also be noted that we have established a likely correlation with reperfusion injury in these samples and that differences in protein abundance following the extended recovery period may be attributable to oxidative stress and recovery from its effects on the pulp tissue.

6.1.6 The Effect of Patient Phenotypes on mRNA and Protein Abundances

Throughout experimental sections two and three we attempted to determine the effect of patient phenotypes on the changes in transcriptomic and proteomic expression of the GOIs. Within each of these sections we established that patient phenotypes may play a potential role in the regulation of expression for several of these GOIs; however, phenotype-associated changes never reached any level of statistical significance due to the low number of pulp samples analysed and the number of proteomic evaluations that returned a null result. Of the phenotypic traits analysed, we found that patient ethnicity and patient tooth morphology had the greatest effect on gene expression and protein abundances. It has been postulated in the literature that patients from varied ethnic backgrounds will respond differently to a number of stimuli but this theory has yet to be confirmed. Apex morphology conversely has been shown in several studies to have a potential effect on the mechanisms by which the pulp would respond and our findings appear to substantiate those already published. Although, as stated previously, we require a greater panel of samples in order to identify the significance of apex morphology on the pulpal response.

When the genes which were affected by patient phenotype were considered and compared against their expression patterns in the other experimental sections, we could find no correlation in mRNA expression or protein abundances in the human dental pulp samples. This does not necessarily mean that there is no correlation between these

experimental groups but rather highlights once again the small scale of this project and the need for many more investigations in order to verify and substantiate our findings and confirm our hypotheses.

6.1.7 Linking Responses of Orthodontically Treated Pulp to the Characterised Responses of Pulp Exposed to Varied Hypoxic Intensities

Following the transcriptomic and proteomic analysis of human dental pulps isolated from teeth that had been treated with routine orthodontic treatment, we determined that there was a potential hypoxic response in teeth that had been treated for a period of two weeks prior to extraction. We then attempted to characterise the angiogenic response of human dental pulp explants exposed to various intensities of hypoxia, as it has been shown that a distinct and defined hypoxic intensity will result in a unique transcriptomic and proteomic response (Yuan *et al.* 2008). This was of interest as the highly vascular human dental pulp has been shown to be a rich source of pluripotent stem cells and has come to the forefront of translational research (d'Aquino *et al.* 2009, Pisciotta *et al.* 2015, Tatullo *et al.* 2015). Characterising the response of this tissue to hypoxia may not only assist in expanding the fields of reparative, restorative and clinical dentistry; but may assist in determining an intervention for pathological disease in a clinical field beyond dentistry.

We then attempted to correlate the angiogenic response of orthodontically treated teeth with the angiogenic response of human dental pulps exposed to: (a) intermittent hypoxia, (b) consecutive exposures to intermittent hypoxia and (c) chronic hypoxia. This was done in order to more fully comprehend and characterise the response of the human dental pulp to routine clinical treatment.

It was identified that the time-course of the orthodontic experiments spanned both the initial (24 to 48 hr) and lag phases (up to 20 days) of routine clinical treatment. It has been shown that during these phases of treatment there is a compression of the vasculature within the PDL which will result in localised ischemia, leakage of blood constituents into the surrounding tissue, tissue hyalanization and cell death (Kitase *et al.* 2009, Miyagawa *et al.* 2009, Ren *et al.* 2008). It has further been postulated that the circulatory damage which occurs in the periodontal vasculature at the “pressure” side of the tooth not only results in localised hypoxia in the PDL, but that this has a knock-on effect on the highly sensitive tissue contained within the pulp chamber of the tooth (Romer *et al.* 2014, Sano *et al.* 2002).

We had anticipated that an application of orthodontic force for a duration of one day would correlate with the transcriptomic and proteomic expression pattern from pulps exposed to intermittent hypoxia or repeated exposure to intermittent hypoxia; that a force duration of three days would correspond with repeated exposure to intermittent hypoxia or chronic hypoxia; and that two weeks of force application would correlate with the expression pattern exhibited by pulps exposed to sustained chronic hypoxia. These assumptions were based upon our knowledge from the current literature, which has outlined the physiological differences between each phase of orthodontic tooth movement.

When mRNA expression patterns and protein abundances were compared, we could find no correlation between orthodontic force durations: (a) one day or (b) three days with any intensity of hypoxic exposure. This was unexpected as the initial phase of orthodontic tooth movement has been shown to involve the rapid displacement of the tooth within the PDL. This process of rapid displacement is thought to involve multiple compression and relief cycles of the periodontal vasculature, which is postulated to result in cycles of ischemia and reperfusion in both the periodontium and subsequently,

the human dental pulp; reperfusion was also expected to result in oxidative stress and reperfusion injury in these tissues.

We had supposed that an orthodontic force applied to teeth for a duration of three days prior to extraction would correlate more closely with the expression trends of pulps exposed to repeated cycles of intermittent hypoxia exposure. We had assumed that this was the most likely association, as at this time-point the tooth is moving from the initial phase of tooth movement, where rapid displacement in the periodontium occurs, into the lag phase where the tooth begins to settle into a new position until provision is made for the tooth to move forward through the alveolar bone. This was not found to be true, as at three days of force application there was a general decrease in the mRNA expression of all of the GOIs, whilst repeated exposures to intermittent hypoxia induced a predominantly positive expression for most of the genes analysed transcriptomically.

We had identified in the previous analysis of the orthodontic experimental data, that following an application of orthodontic force for a period of two weeks, there was an angiogenic response in pulps which included hypoxia/ischemia within the tissue. When the expression trends for both mRNA and protein abundances were compared to those of each distinct hypoxic intensity, we were able to match the trends in the two week orthodontically treated teeth to pulps which were exposed to sustained chronic hypoxia for up to 5 hr and allowed to recover for 30 min.

Whilst we had expected that pulps exposed to an orthodontic force for a period of two weeks would exhibit a similar expression trend to pulps exposed to chronic hypoxia, due to our understanding of the physiology of the lag phase of tooth movement; we were surprised that a chronic hypoxic exposure for such a short duration of time appeared to replicate expression trends observed in tissues which had been treated clinically for a period of two weeks.

We attribute these findings to the nature of the experimental model which was used in order to investigate the effect of hypoxic intensity on the pulp's angiogenic response. In this experimental model, pulps were removed from their tightly encapsulated, highly regulated niche environment, dissected in half and were transferred directly into cell culture medium in a cell culture flask which was then flushed with a gas mix containing 1 % oxygen. Tissues were exposed at all sides to the depleted oxygen solution, including the pulp core as a result of the dissection of the tissue; and perfusion of the tissue by the oxygen depleted medium was far greater than that which would occur physiologically in the pulp's natural environment.

In spite of the irregularity of the experimental model, there appeared to be a direct correlation between the transcriptomic and proteomic expression trends in pulps treated orthodontically for a period of two weeks and pulps exposed to sustained chronic hypoxia. We observed an increase in genes associated with hypoxia and inflammation in both data sets at the transcriptomic level but could only identify the increased expression of VEGFA, one of the protagonists of the angiogenic response, in the chronic hypoxia group. As the perfusion of the tissue by the depleted oxygen solution was greater than expected, we hypothesise that increased VEGFA expression will occur at a later stage in orthodontic tooth movement and that orthodontically treated teeth will follow the same expression trends as those observed in the *in vitro* culture for chronic hypoxia.

When proteomic expression trends were compared there were several homologous results between the chronic hypoxia and two week orthodontically treated datasets. PDGFB, SELE and TEK appeared to behave synonymously between the two experimental treatment types. We have previously discussed the correlation of expression of proteins for vascular stability and leukocyte recruitment with a reoxygenation stage and a potential causative reperfusion injury in the hypoxia

experiments. We assume that the orthodontically treated pulps have also been exposed to a reperfusion stage prior to protein abundance analysis, as pulps appear to have been affected by oxidative stress and the induction of inflammation. We propose that this may have occurred during the isolation of the pulp sample where pulps are removed from their dentine-enamel shell prior to being immersed in RNA preservation solution or being snap-frozen in liquid nitrogen. We cannot state conclusively that this reoxygenation stage occurred physiologically within the lag phase of tooth movement, as the remodelling of the vasculature within the PDL has not yet been characterised according to defined time-points in treatment through time-course investigations; although this would not be an unreasonable cause for these observations.

We have previously mentioned that the induction of inflammation, as a result of reperfusion injury, may lead to coagulation of the microvasculature (Carden and Granger 2000, Granger and Kvietys 2015). This is interesting to note as previous research into the effect of routine orthodontic treatment on the human dental pulp has shown that following an application of orthodontic force, there is an increase in the congestion of microvasculature (Lazzaretti *et al.* 2014, Mostafa *et al.* 1991) and a concurrent reduction in pulpal blood flow (Sano *et al.* 2002, Yamaguchi 2007). Our findings suggest that in response to routine orthodontic force application, for a period of up to two weeks, pulps will experience sustained chronic hypoxia followed by reoxygenation, which will result in a reperfusion injury. However, this has yet to be confirmed *in vivo* and future work is required to validate our hypothesis.

Consideration of the vast number of post-transcriptional and post-translational regulators involved within this system; and further work to visualise protein expression and neovascularisation of the tissue would be needed to make conclusive statements.

Chapter VII

Conclusions and Future Work

7.1 Conclusions

This study has investigated the angiogenic response of the human dental pulp to orthodontic force application at both the transcriptomic and proteomic levels and has attempted to correlate varying intensities of hypoxia with the early stages of orthodontic tooth movement. The following conclusions can be drawn:

1. Identification of appropriate protocols for sample collection, storage, RNA_{total} isolation, quantification and amplification is imperative prior to gene expression analysis in any experimental model; as it is essential that the experimental potential for irreplaceable, low RNA_{total} yielding clinical samples is maximised.
2. The most suitable normaliser (Bustin 2000, Bustin 2002, Bustin *et al.* 2005) for gene expression analysis of a human dental pulp treated in an *in vivo* system, where an orthodontic force is applied to the tooth for two weeks prior to extraction, is constructed from a mean ratio of RPL13A and UBC expression.
3. In order to determine true differences in gene expression, the amplification efficiency for the RT-qPCR reaction of each GOI needs to be considered. Where possible, the reaction efficiency should be calculated based on the ‘decrease per cycle’ theory. Should this not be possible and the ‘constant efficiency’ theory is used, reaction efficiencies should be as close as possible to the maximal value; and must fall in close range of those reactions for the reference genes. By purchasing sets of optimised primers, designed and optimised for unique experimental systems, any over- or under-estimation of mRNA present in the sample may be excluded from further consideration.
4. In addition to the correction for amplification efficiency, we have established that RT-qPCR data from human dental pulp explants requires correction for

biological variability as transcriptomic expression of both reference genes and GOIs fluctuates from patient to patient and between teeth from individual patients; therefore, a baseline needs to be established prior to relative expression analysis. We have identified and clarified the required methodology in order to correct for this biological variability in the RT-qPCR data and have outlined this method in detail in our appendices (**Chapter 8, section 8.2.1**).

5. Orthodontic force application has been shown to have a significant effect on mRNA and protein expression in human dental pulps at 1, 3 and 14 days of treatment. We have established that at the onset of treatment, pulps experience oxidative stress accompanied by inflammation. We suggest that the morphology of mature teeth maintains the inflammatory state and that this is then intensified by an increase in interstitial fluid pressure. At the two week duration of force application, human dental pulps are in a hypoxic and inflammatory condition; and angiogenesis may be initiated in a regulatory response to the stressed condition of the tissue.
6. The use of an Ultra-Turrax® homogeniser is inappropriate for the homogenisation of human dental pulp explants, especially when samples are halved, as per the hypoxia experimental protocol. Whilst we were able to recover high concentrations of total protein from samples, some of the pulp tissue was lost to the homogeniser. We propose that all future work for both RNA_{total} and total protein isolation be carried out using a Dounce tissue homogeniser instead.
7. Quantification of proteins, extracted from human dental pulp explants, by ELISA is not recommended as pulp tissues appear to contain low concentrations of our proteins of interest. Total protein yields from these tissues were high, according to BCA analysis; but in order to quantify low-abundance proteins a

high-sensitivity technique, where normalization for patient variability is available, is recommended. We propose that these analyses are supported by *in situ* hybridisation and microscopy in order to substantiate any findings.

8. Halving pulp samples had no effect on mRNA analysis due to the established use of an RNA amplification system. However, by halving pulp samples in the proteomic analysis, within the hypoxia investigations, the total concentration of the low-abundance proteins present in the homogenate were further reduced. We therefore propose that despite the advantages of minimising biological variability, halving human dental pulp explants is inappropriate for this experimental tissue due to the low RNA_{total} and low protein yields of this tissue.
9. While we can state conclusively that there are changes in expression of the mRNAs of angiogenesis-specific growth factors following force application, we are currently unable to elucidate the mechanisms by which the protein abundances are altered. mRNA expression is not directly correlated to an increase or decrease in protein abundance, as a number of post-transcriptional and post-translational modifications must occur to stabilise mRNA and produce proteins which can be secreted and utilised in physiological and pathological processes; these remain to be considered in our experimental model.
10. The Galvanic probe was shown to be an inefficient tool for the accurate measurement of oxygen concentrations of tissue culture media in the *in vitro* hypoxia experiments. All future work where oxygen concentration needs to be determined should be carried out using a FOXY probe.
11. We have confirmed the *a priori* hypothesis that hypoxic activity will induce a varied response in this tissue, with respect to the intensity of the exposure. Each hypoxic intensity was shown to produce a distinct and unique transcriptomic and

proteomic expression pattern, relative to the other hypoxic intensities investigated.

12. The exposure of human dental pulps to hypoxia, at any intensity, has resulted in an angiogenic response within the tissue. This has confirmed the hypothesis that human dental pulps under hypoxia will endeavour to regulate oxygen concentrations by inducing an angiogenic response. The severity of the angiogenic response appears to be correlated with both the duration and intensity of the hypoxic exposure.
13. Exposure to defined intensities of hypoxia has been shown to have a significant effect on the mRNA and protein expression of angiogenesis-specific growth factors in human dental pulp explants. Following exposure over a time-course of increased hypoxic intensity, there appears to be an increase in inflammation and oxidative stress within the tissues in response to reperfusion injury. Reperfusion injury appears to have very little effect on pulp explants under our defined intensities of intermittent and repeated intermittent hypoxia. The inflammatory responses observed at these hypoxic exposures appear to be true artefacts of the angiogenic response of the pulp and are eased by the restoration of normal oxygen concentrations during the reoxygenation stages. Chronic hypoxia appears to result in the most severe angiogenic response and we attribute this to both the need to reverse oxygen deprivation in order to prevent cell death and tissue necrosis; and to the reperfusion injury which results from a sustained exposure of the tissue to depleted oxygen concentrations.
14. The use of prolonged recovery periods, in order to allow for adequate transcription of the proteins of interest, does not consider the continual modification of mRNA and protein stabilities by homeostasis, post-transcriptional modification or post-translational modification. Any attempt to

correlate protein abundance data from prolonged recoveries to protein abundances in direct response to treatment, must take these factors into consideration during their interpretation of the experimental data; or erroneous conclusions will be drawn.

15. The application of routine orthodontic treatment for a period of two weeks appears to deprive the dental pulp tissue of oxygen in a manner that correlates with sustained chronic hypoxia; and results in a state of inflammation within this tissue. We have yet to determine whether this inflammation is due to: (a) the modification of the interstitial fluid pressure in an unyielding environment in the absence of draining vasculature, (b) oxidative stress induced by reoxygenation injury of the tooth as it moves through the alveolar bone in routine clinical treatment or (c) an artefact from the experimental protocol where tissues could become reoxygenated as they are isolated from their dentine-enamel shells.
16. The experimental model used within the *in vitro* hypoxia experiments appears to alter the true angiogenic response of the tissue to hypoxia, as homeostatic regulatory mechanisms are removed by extracting the pulp from its tightly encapsulated space. Tissues are also injured by dissection during sample preparation and by agitation during the flushing process; this could induce a wound healing and angiogenic response in the tissue. We recommend that investigators gain a greater homology with the physiological environment of the tissue in the experimental model prior to attempting to further characterise the pulp's response to hypoxia.
17. Phenotypic characteristics such as patient ethnicity and tooth apex morphology have been shown to have some effect on the mRNA and protein expression of angiogenesis-specific growth factors in the human dental pulp. These phenotypes need to be investigated further in a greater sample of the general

population in order to identify whether or not these factors play a significant role in the pulp's angiogenic response to orthodontic force application or hypoxic exposure.

18. In all experimental investigations within this thesis, samples were collected in order to satisfy the requirements for obtaining statistical significance of experimental data. We propose that the sample groups were too small in order to gain a comprehensive assessment of the human dental pulp's response to both orthodontic force application and hypoxic exposure. We suggest that in order to more fully characterise the response of this tissue to routine clinical treatment and oxygen deprivation, larger sample groups are used in further investigation.
19. While the majority of the work which was performed in the initial stages of this thesis was carried out in order to optimise the molecular analysis of gene expression within the human dental pulp, in order to characterise the angiogenic response to orthodontic force application; we have established that there is little correlation between mRNA expression and protein abundance in any tissue. This lack of correlation is attributed to the large amount of post-transcriptional and post-translation modification which occurs routinely in physiological and pathological processes. We therefore propose that in order to fully characterise the pulp's response to orthodontic force application, molecular analysis of mRNA expression needs to be considered only after proteins have been investigated and localised in tissues, and molecular regulators such as micro RNA's and small interfering RNA's are considered.

7.2 Future Work

The angiogenic response of the human dental pulp to orthodontic force application remains to be fully characterised. Despite having established thorough working protocols; maximising the experimental potential of a single sample; and confirming gene and protein expression of angiogenesis-specific GOIs in this unique tissue, the original questions posed at the onset of investigation have yet to be answered completely.

While every effort has been made to limit the incidence of bias or variability in gene and protein expression profiles by ensuring that patients come from a mixed racial background, that patient groups were compiled from both genders, and that patient age lies within a narrow range where tooth morphology should be similar; the indisputable extent of variability exhibited at both the inter- and intra-patient levels remains a major cause for hindering the identification of any distinct proteomic expression profiles which may be present as a response to orthodontic treatment in this experimental model. In order to eliminate the effect of inter- and intra- patient variability on transcriptomic data and to identify true trends in gene expression profiles during the process of orthodontic tooth movement, we have clarified a statistical protocol for RT-qPCR data analysis. This has yet to be considered for proteomic studies where ELISA is the investigator's technique of choice. We have determined that normalisation and correction for biological variability is available in conjunction with the use of high-specificity mass spectrometry (Yu *et al.* 2007); however, the lower limits of detection for these assays are greater than those assessed in the present study (Boja and Rodriguez 2012, Burgess *et al.* 2014).

Recent studies using bead based multiplex immunoassays (Khan 2012) and intercalating fluorescence immunoassays (Zhang *et al.* 2006) suggest an increased sensitivity and

may allow detection of chemokines, cytokines and growth factors found in human dental pulp.

A number of investigations have been performed using *in vitro* analysis of both pulp tissues and pulp cells. Despite the numerous experimental techniques that can be applied to cells extracted from a single pulp tissue *in vitro*, we do not advise that an *in vitro* model is used for the purpose of understanding our experimental question. By shifting to an *in vitro* model all histological information that could be gathered in an *in vivo* system is lost; and resultant outgrowth of stem cells would alter the cell population of the original tissue. Removing the pulp from its constraining homeostatically controlled niche environment, which is crucial to its response to treatment, requires manipulations of the original sample: (1) Explants are injured by dissection or collagen is digested in order to create cell populations. (2) Growth factors would have to be added to samples in order to sustain proliferation and prevent cell death. Therefore, we recommend that investigators persist in their use of complete human dental pulp tissues for characterising the response of the pulp to routine clinical treatment. While there are ethical limitations to these studies, investigators should maintain the use of human tissue as animal tissues have been shown to differ in their response (Gerli *et al.* 2010, Mostafa *et al.* 1991, Ramazanzadeh *et al.* 2009.).

While we have established statistically significant differences in mRNA expression in both our orthodontic and hypoxia experiments, following the correction for biological variability; we propose that future work be carried out on a larger sample group in both the transcriptomic and proteomic investigations, as the sample group we investigated here was too small to gain a comprehensive understanding of the pulp's response to force application in the general population. We have assessed only teenagers from a precise geographical location and this is not fully representative of the populous. We

suggest that prior to performing further experiments for transcriptomic analysis; a power analysis is performed in order to establish the sample size that will be required.

Although expanding the sample group would be highly favourable in order to verify our findings, it is extremely costly from the perspective of transcriptomic and proteomic expression analyses. It is also unrealistic to expect a smaller independent study such as this one, where a single orthodontist has had to take on additional patients in order to obtain adequate sample numbers, to be able to collect the number of human dental pulps that would be required to verify these findings. As a single pulp is required for RNA_{total} isolation and another pulp is required for homogenisation for proteomic analysis, we can consider that the human dental pulp is an experimentally limiting sample, as only one technique can be performed successfully per pulp tissue when tissues are to be homogenised.

In order to verify our results, extend the scope of the work and expand the sample group without financially crippling smaller independent laboratories, we propose that a collaboration among a number of dental schools/universities is organised in which standardised protocols for orthodontic force application, sample collection and storage, RNA/protein isolation, RNA/protein quantification, RNA amplification, and RNA/protein expression and data analysis are issued and followed to the letter in order to limit the experimental variability.

In expanding our investigation and the scope of work, we propose that future experiments are performed in a strict time-course from 1 day to 60 days of force application, as we have not been able to determine the pulp's reaction to all phases of orthodontic tooth movement (initial, lag, acceleration and linear). These experiments would help to validate the findings of our hypoxia experiments and enable us to determine whether intermittent hypoxia, repeated exposure to intermittent hypoxia or chronic hypoxia truly occurs in the pulp's response to routine orthodontic treatment;

and after which duration of force application this type of hypoxia occurs. We anticipate some complications with this time-course however, as in routine orthodontic treatment for patients with crowding; premolars are extracted in order for the remaining teeth to move into their place and this would not occur in a 60 day experiment.

We would also like to highlight the use of techniques such as laser capture microdissection, in conjunction with blood endothelial cell markers such as CD34⁺⁺, CD105⁺⁺ or CD146⁺⁺, which may be used to analyse the cellular response of a particular region of the pulp to force application. i.e. The apical portion of the root, which is known to experience hypoxia during tooth movement vs. the coronal portion of the pulp where we have yet to determine whether or not hypoxia infiltrates this tissue. Fluorescence-Activated Cell Sorting (FACS) analysis might also be of interest as varying cell types may respond to treatment in differing manners, e.g. fibroblasts vs. odontoblasts. We also propose that post-transcriptional and post-translational moderators be evaluated as these are known to have a fundamental role in the translation of proteins.

It has also been suggested that lymph angiogenesis is increased during inflammation, in order to reduce the interstitial fluid pressure of the tissue (Gerli *et al.* 2010). While hypoxia is responsible for an increase in new blood vessels and these vessels appear to be present according to Derringer *et al.* (2003, 2004), there has been no consideration that the mRNA expression differences of the GOIs in this study are due to lymph angiogenesis for the homeostatic regulation of the interstitial fluid pressure in inflamed tissue. We propose that in conjunction to assessing the angiogenic changes in response to routine clinical orthodontic treatment, further work is performed to establish the responses of the lymphatic system in this experimental model.

The hypoxia model used in the experiments within this thesis show little correlation with the true physiological environment of the human dental pulp. In order to

characterise the physiological and pathological response of the human dental pulp to any intensity of hypoxic exposure it is critical that researchers attempt to better replicate this niche environment prior to performing any future investigation.

The angiogenic response of the human dental pulp to varied intensities of hypoxia has yet to be fully characterised. Whilst we have identified a number of significant changes in the mRNA expression of several GOIs in these samples, we have yet to gain a confirmation of these findings at the proteomic level. We have argued that by halving pulp samples, in order to prevent further introduction of biological variability, we have sacrificed the integrity of the samples and have altered true expression trends by inducing angiogenesis by wound healing through dissection. In addition to moderating the experimental model, we propose that future work be performed for protein analysis which includes techniques such as microscopy, *in situ* hybridisation and a high-specificity quantification assay which can be used in conjunction with statistical correction methods.

Despite the discrepancies introduced by our experimental model, we have identified that there appears to be a correlation with the duration and intensity of hypoxic exposure with the extent of the potential reperfusion injury which is experienced by these tissues. In order to verify the scale of the injury to the human dental pulp tissues we recommend that further work is carried out for the quantification and localisation of reactive oxygen species which are known to behave as cytotoxic agents within cells and tissues.

Additionally, as we have not observed any significant increase in the transcriptomic or proteomic expression of the growth factor VEGFA (a protagonist of the sprouting angiogenesis signalling cascade) at any of the orthodontic treatment time-points, it may be beneficial for investigators to attempt to identify the mechanism by which this angiogenesis occurs. Intussusceptive angiogenesis has been shown to regulate a number of angiogenic growth factors in the absence of VEGFA and we propose that this be

investigated further by utilising techniques such as electron microscopy following a strict time-course of orthodontic treatment.

Finally, we propose that in order to fully characterise the angiogenic response of the human dental pulp to orthodontic force application, a substantial amount of further work needs to be performed where pulpal blood flow, microvessel coagulation, reactive oxidative species, *in situ* localisation of protein expression for GOIs and inflammatory markers are considered, in conjunction with performing high-specificity quantification for proteins of interest and post-transcriptional and post-translational modifiers. We propose that this work be performed in parallel to the assessment of a time-course of changes to the physiology and vasculature of the PDL as the pulp is dependent on these tissues for physiological and pathological support.

To date, little work has been carried out to determine the effect of the dentine-pulp interaction on the angiogenic response of human dental pulps to orthodontic force application. This could be of worth to investigate, although the experimental model will be difficult to define.

Chapter VIII

Appendix

Due to the large volume of figures and tables enclosed within the appendix, we have included this volume on a compact disc, as per King's College London's publication requirements.

For the purpose of satisfying the requirements outlined by the MIQE Guidelines (*Minimum Information for Publication of Quantitative Real-Time PCR Experiments*) we have enclosed all possible requirements in the enclosed Appendix as Section 1 (Bustin *et al.* 2009).

Appendix Section 2 outlines the protocol for biological variability correction as was previously published by Willems *et al.* (2008). This methodology highlights the protocols which have been omitted in the original publication, which have been clarified by Dr Nora Donaldson, King's College London.

Appendix Section 3 outlines the detailed investigation into mRNA and protein expression patterns performed by Dr Nora Donaldson, King's College London. Summary tables have been compiled within the text and explained in great detail. These analyses are for your perusal should you require any further information.

References

- Abdollahi A, Li M, Ping G, Plathow C, Domhan S, Kiessling F, Lee LB, McMahon G, Grone HJ, Lipson KE *et al* (2005): **Inhibition of platelet-derived growth factor signaling attenuates pulmonary fibrosis.** *J Exp Med*, **201**(6):925-935.
- Abdul Wahab RM, Zainal Ariffin SH, Yeen WW, Ahmad NA, Senafi S (2012): **Molecular markers of dental pulp tissue during orthodontic tooth movement a pilot study.** *ScientificWorldJournal*, **2012**:236427
- Abi-Ramia LB, Stuari AS, Stuari MB, Mendes Ade M (2010): **Effects of low-level laser therapy and orthodontic tooth movement on dental pulps in rats.** *Angle Orthod*, **80**(1):116-122.
- Abraham D, Krenn K, Seebacher G, Paulus P, Klepetko W, Aharinejad S (2004): **Upregulated hypoxia-inducible factor-1 DNA binding activity to the vascular endothelial growth factor-A promoter mediates increased vascular permeability in donor lung grafts.** *Ann Thorac Surg*, **77**(5):1751-1755.
- Abramsson A, Lindblom P, Betsholtz C (2003): **Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors.** *J Clin Invest*, **112**(8):1142-1151
- Albini A, Noonan DM, Ferrari N (2007): **Molecular pathways for cancer angioprevention.** *Clin Cancer Res*, **13**(15 Pt 1):4320-4325
- AlQahtani SJ, Hector MP, Liversidge HM (2010): **Brief Communication: The London Atlas of Human Tooth Development and Eruption.** *Am J Phys Anthropol*, **142**(3):481-490
- Anam MT, Ishika A, Hossain MB, Jesmin (2015): **A meta-analysis of hypoxia inducible factor 1-alpha (HIF1A) gene polymorphisms: association with cancers.** *Biomark Res*, **3**:29.
- Andersen CL, Jensen JL, Orntoft TF (2004): **Normalization of real-time quantitative reverse transcription-PCR data a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets.** *Cancer Res*, **64**(15):5245-5250
- Andrae J, Gallini R, Betsholtz C (2008): **Role of platelet-derived growth factors in physiology and medicine.** *Genes Dev*, **22**(10):1276-1312
- Andrae J, Gouveia L, He L, Betsholtz C (2014): **Characterization of platelet-derived growth factor-A expression in mouse tissues using a lacZ knock-in approach.** *PLoS One*, **9**(8):e105477.
- Andres G, Leali D, Mitola S, Coltrini D, Camozzi M, Corsini M, Belleri M, Hirsch E, Schwendener RA, Christofori G *et al* (2009): **A pro-inflammatory signature mediates FGF2-induced angiogenesis.** *J Cell Mol Med*, **13**(8B):2083-2108
- Andujar MB, Magloire H, Hartmann DJ, Ville G, Grimaud JA (1985): **Early mouse molar root development cellular changes and distribution of fibronectin, laminin and type-IV collagen.** *Differentiation*, **30**(2):111-122

- Angelini DJ, Su Q, Yamaji-Kegan K, Fan C, Skinner JT, Poloczek A, El-Haddad H, Cheadle C, Johns RA (2013): **Hypoxia-induced mitogenic factor (HIMF/FIZZ1/RELMalpha) in chronic hypoxia- and antigen-mediated pulmonary vascular remodeling.** *Respir Res*, **14**:1
- Antypas H, Libberton B, Melican K (2014): **Reducing background cytokine expression in epithelial cells without serum starvation.** *MethodsX* 2014, **1**:251-253.
- Aplin AC, Gelati M, Fogel E, Carnevale E, Nicosia RF (2006): **Angiopoietin-1 and vascular endothelial growth factor induce expression of inflammatory cytokines before angiogenesis.** *Physiol Genomics*, **27**(1):20-28
- Aranha AM, Zhang Z, Neiva KG, Costa CA, Hebling J, Nor JE (2010): **Hypoxia enhances the angiogenic potential of human dental pulp cells.** *J Endod*, **36**(10):1633-1637
- Artese L, Rubini C, Ferrero G, Fioroni M, Santinelli A, Piattelli A (2002): **Vascular endothelial growth factor (VEGF) expression in healthy and inflamed human dental pulps.** *J Endod*, **28**(1):20-23.
- Asikainen TM, Schneider BK, Waleh NS, Clyman RI, Ho WB, Flippin LA, Gunzler V, White CW (2005): **Activation of hypoxia-inducible factors in hyperoxia through prolyl 4-hydroxylase blockade in cells and explants of primate lung.** *Proc Natl Acad Sci U S A*, **102**(29):10212-10217
- Augustin HG, Koh GY, Thurston G, Alitalo K (2009): **Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system.** *Nat Rev Mol Cell Biol*, **10**(3):165-177.
- Ausprunk DH, Folkman J (1977): **Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis.** *Microvasc Res*, **14**(1):53-65.
- Avraamides CJ, Garmy-Susini B, Varner JA (2008): **Integrins in angiogenesis and lymphangiogenesis.** *Nat Rev Cancer*, **8**(8):604-617
- Bader BL, Rayburn H, Crowley D, Hynes RO (1998): **Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all alpha v integrins.** *Cell*, **95**(4):507-519
- Bagis B, Atilla P, Cakar N, Hasanreisoglu U (2007): **Immunohistochemical evaluation of endothelial cell adhesion molecules in human dental pulp: effects of tooth preparation and adhesive application.** *Arch Oral Biol*, **52**(8):705-711.
- Bao F, Wu P, Xiao N, Qiu F, Zeng QP (2012): **Nitric oxide-driven hypoxia initiates synovial angiogenesis, hyperplasia and inflammatory lesions in mice.** *PLoS One*, **7**(3):e34494
- Bar M, Bar D, Lehmann B (2009): **Selection and validation of candidate housekeeping genes for studies of human keratinocytes--review and recommendations.** *J Invest Dermatol*, **129**(3):535-537.
- Barrett TB, Benditt EP (1988): **Platelet-derived growth factor gene expression in human atherosclerotic plaques and normal artery wall.** *Proc Natl Acad Sci U S A*, **85**(8):2810-2814
- Bartlett JM (2002): **Approaches to the analysis of gene expression using mRNA: a technical overview.** *Mol Biotechnol*, **21**(2):149-160
- Baumrind S (1969): **A reconsideration of the propriety of the "pressure-tension" hypothesis.** *Am J Orthod*, **55**(1):12-22. 1.
- Bauss O, Rohling J, Meyer K, Kiliaridis S (2009): **Pulp vitality in teeth suffering trauma during orthodontic therapy.** *Angle Orthod*, **79**(1):166-171.

- Bellafiore M, Battaglia G, Bianco A, Farina F, Palma A, Paoli A (2013): **The involvement of MMP-2 and MMP-9 in heart exercise-related angiogenesis.** *J Transl Med*, **11**:283
- Bentley K, Gerhardt H, Bates PA (2008): **Agent-based simulation of notch-mediated tip cell selection in angiogenic sprout initialisation.** *J Theor Biol*, **250**(1):25-36.
- Ben-Yosef Y, Miller A, Shapiro S, Lahat N (2005): **Hypoxia of endothelial cells leads to MMP-2-dependent survival and death.** *Am J Physiol Cell Physiol*, **289**(5):C1321-1331.
- Bergers G, Song S (2005): **The role of pericytes in blood-vessel formation and maintenance.** *Neuro Oncol*, **7**(4):452-464
- Berggreen E, Haug SR, Mkonyi LE, Bletsa A (2009): **Characterization of the dental lymphatic system and identification of cells immunopositive to specific lymphatic markers.** *Eur J Oral Sci*, **117**(1):34-42
- Bhuvaneswari R, Gan YY, Lucky SS, Chin WW, Ali SM, Soo KC, Olivo M (2008): **Molecular profiling of angiogenesis in hypericin mediated photodynamic therapy.** *Mol Cancer*, **7**:56
- Bjorndal L, Ricucci D (2014): **Pulp Inflammation: From the Reversible Pulpitis to Pulp Necrosis During Caries Progression.** In: *The Dental Pulp: Biology, Pathology and Regenerative Therapies*. Edited by Goldberg M. Heidelberg, New York, Dordrecht, London: Springer-Verlag Berlin Heidelberg; 125-140.
- Boja ES, Rodriguez H (2012): **Mass spectrometry-based targeted quantitative proteomics: achieving sensitive and reproducible detection of proteins.** *Proteomics*, **12**(8):1093-1110.
- Bondjers C, Kalen M, Hellstrom M, Scheidl SJ, Abramsson A, Renner O, Lindahl P, Cho H, Kehrl J, Betsholtz C (2003): **Transcription profiling of platelet-derived growth factor-B-deficient mouse embryos identifies RGS5 as a novel marker for pericytes and vascular smooth muscle cells.** *Am J Pathol*, **162**(3):721-729.
- Bonnet S, Michelakis ED, Porter CJ, Andrade-Navarro MA, Thebaud B, Haromy A, Harry G, Moudgil R, McMurtry MS, Weir EK *et al* (2006): **An abnormal mitochondrial-hypoxia inducible factor-1 α -Kv channel pathway disrupts oxygen sensing and triggers pulmonary arterial hypertension in fawn hooded rats: similarities to human pulmonary arterial hypertension.** *Circulation*, **113**(22):2630-2641.
- Booth CS, Pienaar E, Termaat JR, Whitney SE, Louw TM, Viljoen HJ (2010): **Efficiency of the Polymerase Chain Reaction.** *Chem Eng Sci*, **65**(17):4996-5006
- Borovecki F, Lovrecic L, Zhou J, Jeong H, Then F, Rosas HD, Hersch SM, Hogarth P, Bouzou B, Jensen RV *et al* (2005): **Genome-wide expression profiling of human blood reveals biomarkers for Huntington's disease.** *Proc Natl Acad Sci U S A*, **102**(31):11023-11028
- Branco-Price C, Evans CE, Johnson RS (2013): **Endothelial hypoxic metabolism in carcinogenesis and dissemination: HIF-A isoforms are a NO metastatic phenomenon.** *Oncotarget*, **4**(12):2567-2576
- Breton-Romero R, Lamas S (2014): **Hydrogen peroxide signaling in vascular endothelial cells.** *Redox Biol*, **2**:529-534
- Brindle NP, Saharinen P, Alitalo K (2006): **Signaling and functions of angiopoietin-1 in vascular protection.** *Circ Res*, **98**(8):1014-1023.

- Brogi E, Wu T, Namiki A, Isner JM (1994): **Indirect angiogenic cytokines upregulate VEGF and bFGF gene expression in vascular smooth muscle cells, whereas hypoxia upregulates VEGF expression only.** *Circulation*, **90**(2):649-652.
- Brooks PC, Stromblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG, Quigley JP, Cheresch DA (1996): **Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3.** *Cell*, **85**(5):683-693.
- Brown LF, Yeo KT, Berse B, Yeo TK, Senger DR, Dvorak HF, van de Water L (1992): **Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing.** *J Exp Med*, **176**(5):1375-1379.
- Brunner D, Frank J, Appl H, Schoffl H, Pfaller W, Gstraunthaler G (2010): **Serum-free cell culture: the serum-free media interactive online database.** *ALTEX*, **27**(1):53-62
- Buchaille R, Couble ML, Magloire H, Bleicher F (2000): **A subtractive PCR-based cDNA library from human odontoblast cells: identification of novel genes expressed in tooth forming cells.** *Matrix Biol*, **19**(5):421-430.
- Burgess MW, Keshishian H, Mani DR, Gillette MA, Carr SA (2014): **Simplified and efficient quantification of low-abundance proteins at very high multiplex via targeted mass spectrometry.** *Mol Cell Proteomics*, **13**(4):1137-1149.
- Burns JS, Kristiansen M, Kristensen LP, Larsen KH, Nielsen MO, Christiansen H, Nehlin J, Andersen JS, Kassem M (2011): **Decellularized matrix from tumorigenic human mesenchymal stem cells promotes neovascularization with galectin-1 dependent endothelial interaction.** *PLoS One*, **6**(7):e21888
- Burri PH, Djonov V (2002): **Intussusceptive angiogenesis--the alternative to capillary sprouting.** *Mol Aspects Med*, **23**(6S):S1-27
- Burri PH, Hlushchuk R, Djonov V (2004): **Intussusceptive angiogenesis: its emergence, its characteristics, and its significance.** *Dev Dyn*, **231**(3):474-488.
- Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL *et al* (2009): **The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments.** *Clin Chem*, **55**(4):611-622.
- Bustin SA, Benes V, Nolan T, Pfaffl MW (2005): **Quantitative real-time RT-PCR--a perspective.** *J Mol Endocrinol*, **34**(3):597-601.
- Bustin SA, Nolan T (2004): **Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction.** *J Biomol Tech*, **15**(3):155-166.
- Bustin SA (2000): **Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays.** *J Mol Endocrinol*, **25**(2):169-193. Fleige S, Pfaffl MW: **RNA integrity and the effect on the real-time qRT-PCR performance.** *Mol Aspects Med* 2006, **27**(2-3):126-139
- Bustin SA (2002): **Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems.** *J Mol Endocrinol*, **29**(1):23-39
- Bustin SA (2005): **Real-time, fluorescence-based quantitative PCR: a snapshot of current procedures and preferences.** *Expert Rev Mol Diagn* 2005, **5**(4):493-498.
- Bustin SA (2010): **Why the need for qPCR publication guidelines?--The case for MIQE.** *Methods*, **50**(4):217-226.

- Carden DL, Granger DN (2000): **Pathophysiology of ischaemia-reperfusion injury.** *J Pathol*, **190**(3):255-266.
- Carmeliet P, Collen D (1998): **Vascular development and disorders: molecular analysis and pathogenic insights.** *Kidney Int*, **53**(6):1519-1549
- Carmeliet P (2000): **Mechanisms of angiogenesis and arteriogenesis.** *Nat Med*, **6**(4):389-395
- Caruana GaM, R. (2004): **Assessing genomic DNA contamination of total RNA isolated from kidney cells obtained by Laser Capture Microdissection using the Agilent RNA 6000 Pico assay.** In.: Agilent Technologies;: 1-4.
- Chappell JC, Wiley DM, Bautch VL (2011): **Regulation of blood vessel sprouting.** *Semin Cell Dev Biol*, **22**(9):1005-1011
- Chung JC, Shum-Tim D (2012): **Neovascularization in tissue engineering.** *Cells*, **1**(4):1246-1260
- Ciucan L, Bonneau O, Hussey M, Duggan N, Holmes AM, Good R, Stringer R, Jones P, Morrell NW, Jarai G *et al* (2011): **A novel murine model of severe pulmonary arterial hypertension.** *Am J Respir Crit Care Med*, **184**(10):1171-1182
- Couble ML, Farges JC, Bleicher F, Perrat-Mabillon B, Boudeulle M, Magloire H (2000): **Odontoblast differentiation of human dental pulp cells in explant cultures.** *Calcif Tissue Int*, **66**(2):129-138.
- Coulson DT, Brockbank S, Quinn JG, Murphy S, Ravid R, Irvine GB, Johnston JA (2008): **Identification of valid reference genes for the normalization of RT qPCR gene expression data in human brain tissue.** *BMC Mol Biol* 2008, **9**:46.
- Cowburn AS, Takeda N, Boutin AT, Kim JW, Sterling JC, Nakasaki M, Southwood M, Goldrath AW, Jamora C, Nizet V *et al* (2013): **HIF isoforms in the skin differentially regulate systemic arterial pressure.** *Proc Natl Acad Sci U S A*, **110**(43):17570-17575
- Cremer FW, Kiel K, Wallmeier M, Goldschmidt H, Moos M (1997): **A quantitative PCR assay for the detection of low amounts of malignant cells in multiple myeloma.** *Ann Oncol*, **8**(7):633-636.
- Dale EA, Ben Mabrouk F, Mitchell GS (2014): **Unexpected benefits of intermittent hypoxia: enhanced respiratory and nonrespiratory motor function.** *Physiology (Bethesda)*, **29**(1):39-48
- d'Aquino R, De Rosa A, Laino G, Caruso F, Guida L, Rullo R, Checchi V, Laino L, Tirino V, Papaccio G (2009): **Human dental pulp stem cells: from biology to clinical applications.** *J Exp Zool B Mol Dev Evol* 2009, **312B**(5):408-415.
- D'Asti E, Kool M, Pfister SM, Rak J (2014): **Coagulation and angiogenic gene expression profiles are defined by molecular subgroups of medulloblastoma: evidence for growth factor-thrombin cross-talk.** *J Thromb Haemost*, **12**(11):1838-1849
- Davidovitch Z, Finkelson MD, Steigman S, Shanfeld JL, Montgomery PC, Korostoff E (1980): **Electric currents, bone remodeling, and orthodontic tooth movement. II. Increase in rate of tooth movement and periodontal cyclic nucleotide levels by combined force and electric current.** *Am J Orthod*, **77**(1):33-47
- Davidovitch Z, Nicolay OF, Ngan PW, Shanfeld JL (1988): **Neurotransmitters, cytokines, and the control of alveolar bone remodeling in orthodontics.** *Dent Clin North Am*, **32**(3):411-435.

- Davoren PA, McNeill RE, Lowery AJ, Kerin MJ, Miller N (2008): **Identification of suitable endogenous control genes for microRNA gene expression analysis in human breast cancer.** *BMC Mol Biol*, **9**:76.
- de Kok JB, Roelofs RW, Giesendorf BA, Pennings JL, Waas ET, Feuth T, Swinkels DW, Span PN (2005): **Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes.** *Lab Invest*, **85**(1):154-159
- De Leon H, Boue S, Schlage WK, Boukharov N, Westra JW, Gebel S, VanHooser A, Talikka M, Fields RB, Veljkovic E *et al* (2014): **A vascular biology network model focused on inflammatory processes to investigate atherogenesis and plaque instability.** *J Transl Med*, **12**:185
- De Spiegelaere W, Casteleyn C, Van den Broeck W, Plendl J, Bahramsoltani M, Simoens P, Djonov V, Cornillie P (2012): **Intussusceptive angiogenesis: a biologically relevant form of angiogenesis.** *J Vasc Res* 2012, **49**(5):390-404.
- Delrieu I (2000): **The high molecular weight isoforms of basic fibroblast growth factor (FGF-2): an insight into an intracrine mechanism.** *FEBS Lett*, **468**(1):6-10
- Deng DX, Spin JM, Tsalenko A, Vailaya A, Ben-Dor A, Yakhini Z, Tsao P, Bruhn L, Quertermous T (2006a): **Molecular signatures determining coronary artery and saphenous vein smooth muscle cell phenotypes: distinct responses to stimuli.** *Arterioscler Thromb Vasc Biol*, **26**(5):1058-1065
- Deng DX, Tsalenko A, Vailaya A, Ben-Dor A, Kundu R, Estay I, Tabibiazar R, Kincaid R, Yakhini Z, Bruhn L *et al* (2006b): **Differences in vascular bed disease susceptibility reflect differences in gene expression response to atherogenic stimuli.** *Circ Res*, **98**(2):200-208
- Dengler VL, Galbraith MD, Espinosa JM (2014): **Transcriptional regulation by hypoxia inducible factors.** *Crit Rev Biochem Mol Biol*, **49**(1):1-15.
- Derringer KA, Jagers DC, Linden RW (1996): **Angiogenesis in human dental pulp following orthodontic tooth movement.** *J Dent Res*, **75**(10):1761-1766.
- Derringer KA, Linden RW (2003): **Angiogenic growth factors released in human dental pulp following orthodontic force.** *Arch Oral Biol*, **48**(4):285-291.
- Derringer KA, Linden RW (1998): **Enhanced angiogenesis induced by diffusible angiogenic growth factors released from human dental pulp explants of orthodontically moved teeth.** *Eur J Orthod*, **20**(4):357-367.
- Derringer KA, Linden RW (2007): **Epidermal growth factor released in human dental pulp following orthodontic force.** *Eur J Orthod*, **29**(1):67-71
- Derringer KA, Linden RW (2004): **Vascular endothelial growth factor, fibroblast growth factor 2, platelet derived growth factor and transforming growth factor beta released in human dental pulp following orthodontic force.** *Arch Oral Biol*, **49**(8):631-641
- Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A (2004): **Validation of housekeeping genes for normalizing RNA expression in real-time PCR.** *Biotechniques*, **37**(1):112-114, 116, 118-119.
- Di Pietro M, Parborell F, Irusta G, Pascuali N, Bas D, Bianchi MS, Tesone M, Abramovich D (2015): **Metformin regulates ovarian angiogenesis and follicular development in a female polycystic ovary syndrome rat model.** *Endocrinology*, **156**(4):1453-1463

- Digka A, Lyroudia K, Jirasek T, Kasampalidis IN, Karayannopoulou G, Kubinova L (2006): **Visualisation of human dental pulp vasculature by immunohistochemical and immunofluorescent detection of CD34: a comparative study.** *Aust Endod J*, **32**(3):101-106.
- Dinarello CA, Fossati G, Mascagni P (2011): **Histone deacetylase inhibitors for treating a spectrum of diseases not related to cancer.** *Mol Med*, **17**(5-6):333-352
- Dirks RP, Bloemers HP (1995): **Signals controlling the expression of PDGF.** *Mol Biol Rep*, **22**(1):1-24.
- Dissanayaka WL, Zhu L, Hargreaves KM, Jin L, Zhang C (2015): **In vitro analysis of scaffold-free prevascularized microtissue spheroids containing human dental pulp cells and endothelial cells.** *J Endod*, **41**(5):663-670.
- Djonov V, Schmid M, Tschanz SA, Burri PH (2000): **Intussusceptive angiogenesis: its role in embryonic vascular network formation.** *Circ Res*, **86**(3):286-292.
- Dong Y, Lan W, Wu W, Huang Z, Zhao J, Peng L, Wang J (2013): **Increased expression of EphA7 in inflamed human dental pulp.** *J Endod*, **39**(2):223-227.
- Dorland WAN (2007): **Dorland's Medical Dictionary for Health Consumers.** In.: W B Saunders, Elsevier.
- Dorow C, Krstin N, Sander FG (2002): **Experiments to determine the material properties of the periodontal ligament.** *J Orofac Orthop*, **63**(2):94-104.
- Eisenberg E, Levanon EY (2003): **Human housekeeping genes are compact.** *Trends Genet*, **19**(7):362-365.
- ElAli A, Theriault P, Rivest S (2014): **The role of pericytes in neurovascular unit remodeling in brain disorders.** *Int J Mol Sci*, **15**(4):6453-6474.
- Elks PM, Brizee S, van der Vaart M, Walmsley SR, van Eeden FJ, Renshaw SA, Meijer AH (2013): **Hypoxia inducible factor signaling modulates susceptibility to mycobacterial infection via a nitric oxide dependent mechanism.** *PLoS Pathog*, **9**(12):e1003789
- Eltzschig HK, Collard CD (2004): **Vascular ischaemia and reperfusion injury.** *Br Med Bull*, **70**:71-86
- Fan L, Li J, Yu Z, Dang X, Wang K (2014): **The hypoxia-inducible factor pathway, prolyl hydroxylase domain protein inhibitors, and their roles in bone repair and regeneration.** *Biomed Res Int*, **2014**:239356
- Farahani RM, Xaymardan M (2015): **Platelet-Derived Growth Factor Receptor Alpha as a Marker of Mesenchymal Stem Cells in Development and Stem Cell Biology.** *Stem Cells Int*, **2015**:362753
- Fecteau KA, Mrkonjich L, Mason JI, Mendis-Handagama SM (2006): **Detection of platelet-derived growth factor-alpha (PDGF-A) protein in cells of Leydig lineage in the postnatal rat testis.** *Histol Histopathol*, **21**(12):1295-1302.
- Felaco M, Di Maio FD, De Fazio P, D'Arcangelo C, De Lutiis MA, Varvara G, Grilli A, Barbacane RC, Reale M, Conti P (2000): **Localization of the e-NOS enzyme in endothelial cells and odontoblasts of healthy human dental pulp.** *Life Sci*, **68**(3):297-306.

- Ferrari G, Cook BD, Terushkin V, Pintucci G, Mignatti P (2009): **Transforming growth factor-beta 1 (TGF-beta1) induces angiogenesis through vascular endothelial growth factor (VEGF)-mediated apoptosis.** *J Cell Physiol*, **219**(2):449-458
- Fink T, Lund P, Pilgaard L, Rasmussen JG, Duroux M, Zachar V (2008): **Instability of standard PCR reference genes in adipose-derived stem cells during propagation, differentiation and hypoxic exposure.** *BMC Mol Biol*, **9**:98.
- Flamme I, Frohlich T, von Reutern M, Kappel A, Damert A, Risau W (1997): **HRF, a putative basic helix-loop-helix-PAS-domain transcription factor is closely related to hypoxia-inducible factor-1 alpha and developmentally expressed in blood vessels.** *Mech Dev*, **63**(1):51-60.
- Fleige S, Pfaffl MW (2006): **RNA integrity and the effect on the real-time qRT-PCR performance.** *Mol Aspects Med*, **27**(2-3):126-139.
- Fleige S, Walf V, Huch S, Prgomet C, Sehm J, Pfaffl MW (2006): **Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR.** *Biotechnol Lett*, **28**(19):1601-1613.
- Floege J, Eitner F, Alpers CE (2008): **A new look at platelet-derived growth factor in renal disease.** *J Am Soc Nephrol*, **19**(1):12-23.
- Florell SR, Coffin CM, Holden JA, Zimmermann JW, Gerwels JW, Summers BK, Jones DA, Leachman SA (2001): **Preservation of RNA for functional genomic studies: a multidisciplinary tumor bank protocol.** *Mod Pathol*, **14**(2):116-128.
- Folkman J, D'Amore PA (1996): **Blood vessel formation: what is its molecular basis?** *Cell*, **87**(7):1153-1155
- Folkman J, Shing Y (1992): **Angiogenesis.** *J Biol Chem*, **267**(16):10931-10934.
- Forget MA, Voorhees JL, Cole SL, Dakhallallah D, Patterson IL, Gross AC, Moldovan L, Mo X, Evans R, Marsh CB *et al* (2014): **Macrophage colony-stimulating factor augments Tie2-expressing monocyte differentiation, angiogenic function, and recruitment in a mouse model of breast cancer.** *PLoS One*, **9**(6):e98623
- Fraser ST (2013): **The modern primitives: applying new technological approaches to explore the biology of the earliest red blood cells.** *ISRN Hematol* 2013, **2013**:568928
- Freeman WM, Walker SJ, Vrana KE (1999): **Quantitative RT-PCR: pitfalls and potential.** *Biotechniques*, **26**(1):112-122, 124-115.
- Fujio M, Xing Z, Sharabi N, Xue Y, Yamamoto A, Hibi H, Ueda M, Fristad I, Mustafa K (2015): **Conditioned media from hypoxic-cultured human dental pulp cells promotes bone healing during distraction osteogenesis.** *J Tissue Eng Regen Med*.
- Fukuhara S, Sako K, Noda K, Zhang J, Minami M, Mochizuki N (2010): **Angiopoietin-1/Tie2 receptor signaling in vascular quiescence and angiogenesis.** *Histol Histopathol*, **25**(3):387-396.
- Gallego Romero I, Pai AA, Tung J, Gilad Y (2014): **RNA-seq: impact of RNA degradation on transcript quantification.** *BMC Biol*, **12**:42.
- Gebala V, Collins R, Geudens I, Phng LK, Gerhardt H (2016): **Blood flow drives lumen formation by inverse membrane blebbing during angiogenesis in vivo.** *Nat Cell Biol*, **18**(4):443-450.
- Gene Quantification Website - Source for Housekeeping Gene Validation Softwares
[<http://normalisation.gene-quantification.info/>]

- Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D *et al* (2003): **VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia.** *J Cell Biol*, **161**(6):1163-1177
- Gerhardt H (2000-2013): **VEGF and Endothelial Guidance in Angiogenic Sprouting.** . In: *Madame Curie Bioscience Database [Internet]*. Austin, TX: Landes Bioscience.
- Gerli R, Secciani I, Sozio F, Rossi A, Weber E, Lorenzini G (2010): **Absence of lymphatic vessels in human dental pulp: a morphological study.** *Eur J Oral Sci*, **118**(2):110-117.
- Gianni-Barrera R, Bartolomeo M, Vollmar B, Djonov V, Banfi A (2014): **Split for the cure: VEGF, PDGF-BB and intussusception in therapeutic angiogenesis.** *Biochem Soc Trans*, **42**(6):1637-1642.
- Giatromanolaki A, Koukourakis MI, Pezzella F, Sivridis E, Turley H, Harris AL, Gatter KC (2008): **Phosphorylated VEGFR2/KDR receptors are widely expressed in B-cell non-Hodgkin's lymphomas and correlate with hypoxia inducible factor activation.** *Hematol Oncol*, **26**(4):219-224
- Gilles C, Polette M, Piette J, Munaut C, Thompson EW, Birembaut P, Foidart JM (1996): **High level of MT-MMP expression is associated with invasiveness of cervical cancer cells.** *Int J Cancer*, **65**(2):209-213.
- Glesne DA, Zhang W, Mandava S, Ursos L, Buell ME, Makowski L, Rodi DJ (2006): **Subtractive transcriptomics: establishing polarity drives in vitro human endothelial morphogenesis.** *Cancer Res*, **66**(8):4030-4040
- Glockner S, Lehmann U, Wilke N, Kleeberger W, Langer F, Kreipe H (2000): **Detection of gene amplification in intraductal and infiltrating breast cancer by laser-assisted microdissection and quantitative real-time PCR.** *Pathobiology*, **68**(4-5):173-179
- Goel S, Duda DG, Xu L, Munn LL, Boucher Y, Fukumura D, Jain RK (2011): **Normalization of the vasculature for treatment of cancer and other diseases.** *Physiol Rev*, **91**(3):1071-1121.
- Goidin D, Mamessier A, Staquet MJ, Schmitt D, Berthier-Vergnes O (2001): **Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and beta-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations.** *Anal Biochem*, **295**(1):17-21.
- Gomm JJ, Browne PJ, Coope RC, Bansal GS, Yiangou C, Johnston CL, Mason R, Coombes RC (1997): **A paracrine role for myoepithelial cell-derived FGF2 in the normal human breast.** *Exp Cell Res*, **234**(1):165-173
- Granger DN, Kvietys PR (2015): **Reperfusion injury and reactive oxygen species: The evolution of a concept.** *Redox Biol*, **6**:524-551.
- Grasemann H, Knauer N, Buscher R, Hubner K, Drazen JM, Ratjen F (2000): **Airway nitric oxide levels in cystic fibrosis patients are related to a polymorphism in the neuronal nitric oxide synthase gene.** *Am J Respir Crit Care Med*, **162**(6):2172-2176
- Gronthos S, Brahimi J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S (2002): **Stem cell properties of human dental pulp stem cells.** *J Dent Res*, **81**(8):531-535
- Grotzer MA, Patti R, Geoerger B, Eggert A, Chou TT, Phillips PC (2000): **Biological stability of RNA isolated from RNAlater-treated brain tumor and neuroblastoma xenografts.** *Med Pediatr Oncol*, **34**(6):438-442.

- Grunheid T, Morbach BA, Zentner A (2007): **Pulpal cellular reactions to experimental tooth movement in rats.** *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, **104**(3):434-441.
- Gstraunthaler G (2003): **Alternatives to the use of fetal bovine serum: serum-free cell culture.** *ALTEX*, **20**(4):275-281.
- Gubern C, Hurtado O, Rodriguez R, Morales JR, Romera VG, Moro MA, Lizasoain I, Serena J, Mallolas J (2009): **Validation of housekeeping genes for quantitative real-time PCR in in-vivo and in-vitro models of cerebral ischaemia.** *BMC Mol Biol*, **10**:57.
- Hadjipanayi E, Schilling AF (2013): **Hypoxia-based strategies for angiogenic induction: the dawn of a new era for ischemia therapy and tissue regeneration.** *Organogenesis*, **9**(4):261-272
- Hahn CL, Liewehr FR (2007): **Update on the adaptive immune responses of the dental pulp.** *J Endod*, **33**(7):773-781
- Hall CJ, Freer TJ (1998): **The effects of early orthodontic force application on pulp test responses.** *Aust Dent J*, **43**(5):359-361.
- Hamersky PA, Weimer AD, Taintor JF (1980): **The effect of orthodontic force application on the pulpal tissue respiration rate in the human premolar.** *Am J Orthod*, **77**(4):368-378
- Han G, Chen Y, Hou J, Liu C, Chen C, Zhuang J, Meng W (2010): **Effects of simvastatin on relapse and remodeling of periodontal tissues after tooth movement in rats.** *Am J Orthod Dentofacial Orthop*, **138**(5):550 e551-557; discussion 550-551.
- Han G, Hu M, Zhang Y, Jiang H (2013): **Pulp vitality and histologic changes in human dental pulp after the application of moderate and severe intrusive orthodontic forces.** *Am J Orthod Dentofacial Orthop*, **144**(4):518-522.
- Hanjaya-Putra D, Bose V, Shen YI, Yee J, Khetan S, Fox-Talbot K, Steenbergen C, Burdick JA, Gerecht S (2011): **Controlled activation of morphogenesis to generate a functional human microvasculature in a synthetic matrix.** *Blood*, **118**(3):804-815
- Hansen MC, Nielsen AK, Molin S, Hammer K, Kilstrup M (2001): **Changes in rRNA levels during stress invalidates results from mRNA blotting: fluorescence in situ rRNA hybridization permits renormalization for estimation of cellular mRNA levels.** *J Bacteriol*, **183**(16):4747-4751.
- Hayashi H, Sano H, Seo S, Kume T (2008): **The Foxc2 transcription factor regulates angiogenesis via induction of integrin beta3 expression.** *J Biol Chem*, **283**(35):23791-23800
- Heck TAM, Vaeyens MM, Van Oosterwyck H (2015): **Computational Models of Sprouting Angiogenesis and Cell Migration: Towards Multiscale Mechanochemical Models of Angiogenesis.** *Mathematical Modelling of Natural Phenomena*, **10**(1):108-141.
- Heid CA, Stevens J, Livak KJ, Williams PM (1996): **Real time quantitative PCR.** *Genome Res*, **6**(10):986-994.
- Heinzman JM, Brower SL, Bush JE (2008): **Comparison of angiogenesis-related factor expression in primary tumor cultures under normal and hypoxic growth conditions.** *Cancer Cell Int*, **8**:11

- Heldin CH, Ernlund A, Rorsman C, Ronnstrand L (1989): **Dimerization of B-type platelet-derived growth factor receptors occurs after ligand binding and is closely associated with receptor kinase activation.** *J Biol Chem*, **264**(15):8905-8912
- Heldin CH, Westermark B (1999): **Mechanism of action and in vivo role of platelet-derived growth factor.** *Physiol Rev*, **79**(4):1283-1316.
- Henneman S, Von den Hoff JW, Maltha JC (2008): **Mechanobiology of tooth movement.** *Eur J Orthod*, **30**(3):299-306.
- Heuchel R, Berg A, Tallquist M, Ahlen K, Reed RK, Rubin K, Claesson-Welsh L, Heldin CH, Soriano P (1999): **Platelet-derived growth factor beta receptor regulates interstitial fluid homeostasis through phosphatidylinositol-3' kinase signaling.** *Proc Natl Acad Sci U S A*, **96**(20):11410-11415.
- Heyeraas KJ, Berggreen E (1999): **Interstitial fluid pressure in normal and inflamed pulp.** *Crit Rev Oral Biol Med*, **10**(3):328-336.
- Hicklin DJ, Ellis LM (2005): **Role of the Vascular Endothelial Growth Factor Pathway in Tumor Growth and Angiogenesis.** *Journal of Clinical Oncology*, **23**(5):1011-1027
- Hirota K, Semenza GL (2006): **Regulation of angiogenesis by hypoxia-inducible factor 1.** *Crit Rev Oncol Hematol*, **59**(1):15-26.
- Hjort MR, Brenyo AJ, Finkelstein JN, Frampton MW, LoMonaco MB, Stewart JC, Johnston CJ, D'Angio CT (2003): **Alveolar epithelial cell-macrophage interactions affect oxygen-stimulated interleukin-8 release.** *Inflammation*, **27**(3):137-145
- Hoch RV, Soriano P (2003): **Roles of PDGF in animal development.** *Development*, **130**(20):4769-4784.
- Hodivala-Dilke KM, McHugh KP, Tsakiris DA, Rayburn H, Crowley D, Ullman-Cullere M, Ross FP, Collier BS, Teitelbaum S, Hynes RO (1999): **Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival.** *J Clin Invest*, **103**(2):229-238.
- Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA (2004): **Vascular endothelial growth factor and angiogenesis.** *Pharmacol Rev*, **56**(4):549-580.
- Hosford GE, Olson DM (2003): **Effects of hyperoxia on VEGF, its receptors, and HIF-2alpha in the newborn rat lung.** *Am J Physiol Lung Cell Mol Physiol*, **285**(1):L161-168
- Hsiao LL, Dangond F, Yoshida T, Hong R, Jensen RV, Misra J, Dillon W, Lee KF, Clark KE, Haverty P *et al* (2001): **A compendium of gene expression in normal human tissues.** *Physiol Genomics*, **7**(2):97-104
- <http://www.genome.jp/kegg/>
- <http://www.ndexbio.org/>
- Huang GT, Potente AP, Kim JW, Chugal N, Zhang X (1999): **Increased interleukin-8 expression in inflamed human dental pulps.** *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, **88**(2):214-220
- Huang GT, Sonoyama W, Chen J, Park SH (2006): **In vitro characterization of human dental pulp cells: various isolation methods and culturing environments.** *Cell Tissue Res*, **324**(2):225-236.
- Huang H, Bhat A, Woodnutt G, Lappe R (2010): **Targeting the ANGPT-TIE2 pathway in malignancy.** *Nat Rev Cancer*, **10**(8):575-585

- Huggett J, Dheda K, Bustin S, Zumla A (2005): **Real-time RT-PCR normalisation; strategies and considerations.** *Genes Immun*, **6**(4):279-284.
- Imbeaud S, Graudens E, Boulanger V, Barlet X, Zaborski P, Eveno E, Mueller O, Schroeder A, Auffray C (2005): **Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces.** *Nucleic Acids Res*, **33**(6):e56.
- Ing NH (2005): **Steroid hormones regulate gene expression posttranscriptionally by altering the stabilities of messenger RNAs.** *Biol Reprod*, **72**(6):1290-1296.
- Ishikawa K, Yoshida S, Kadota K, Nakamura T, Niirō H, Arakawa S, Yoshida A, Akashi K, Ishibashi T (2010): **Gene expression profile of hyperoxic and hypoxic retinas in a mouse model of oxygen-induced retinopathy.** *Invest Ophthalmol Vis Sci*, **51**(8):4307-4319
- Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG, Jr. (2001): **HIF α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing.** *Science*, **292**(5516):464-468.
- Iwashina M, Shichiri M, Marumo F, Hirata Y (1998): **Transfection of inducible nitric oxide synthase gene causes apoptosis in vascular smooth muscle cells.** *Circulation*, **98**(12):1212-1218
- Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, von Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ *et al* (2001): **Targeting of HIF- α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation.** *Science*, **292**(5516):468-472
- Jakobsson L, Franco CA, Bentley K, Collins RT, Ponsioen B, Aspalter IM, Rosewell I, Busse M, Thurston G, Medvinsky A *et al* (2010): **Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting.** *Nat Cell Biol*, **12**(10):943-953.
- Javed F, Al-Kheraif AA, Romanos EB, Romanos GE (2015): **Influence of orthodontic forces on human dental pulp: a systematic review.** *Arch Oral Biol*, **60**(2):347-356.
- Jeffreys AJ, Wilson V, Neumann R, Keyte J (1988): **Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells.** *Nucleic Acids Res*, **16**(23):10953-10971.
- Jiang YZ, Li Y, Wang K, Dai CF, Huang SA, Chen DB, Zheng J (2014): **Distinct roles of HIF1A in endothelial adaptations to physiological and ambient oxygen.** *Mol Cell Endocrinol*, **391**(1-2):60-67
- Johnson M (2012): **Protein Quantitation.** *Master Methods*, **2**
- Jones CA, London NR, Chen H, Park KW, Sauvaget D, Stockton RA, Wythe JD, Suh W, Larrieu-Lahargue F, Mukoyama YS *et al* (2008): **Robo4 stabilizes the vascular network by inhibiting pathologic angiogenesis and endothelial hyperpermeability.** *Nat Med*, **14**(4):448-453
- Jones LJ, Yue ST, Cheung CY, Singer VL (1998): **RNA quantitation by fluorescence-based solution assay. RiboGreen reagent characterization.** *Anal Biochem*, **265**(2):368-374.
- Jung F, Palmer LA, Zhou N, Johns RA (2000): **Hypoxic regulation of inducible nitric oxide synthase via hypoxia inducible factor-1 in cardiac myocytes.** *Circ Res*, **86**(3):319-325. Aktan F: **iNOS-mediated nitric oxide production and its regulation.** *Life Sci* 2004, **75**(6):639-653.

- Kaetzel DM (2003): **Transcription of the platelet-derived growth factor A-chain gene.** *Cytokine Growth Factor Rev*, **14**(5):427-446.
- Kalogeris T, Baines CP, Krenz M, Korthuis RJ (2012): **Cell biology of ischemia/reperfusion injury.** *Int Rev Cell Mol Biol*, **298**:229-317.
- Katayose D, Ohe M, Yamauchi K, Ogata M, Shirato K, Fujita H, Shibahara S, Takishima T (1993): **Increased expression of PDGF A- and B-chain genes in rat lungs with hypoxic pulmonary hypertension.** *Am J Physiol*, **264**(2 Pt 1):L100-106
- Kawase J, Ozawa S, Kobayashi K, Imaeda Y, Umemoto S, Matsumoto S, Ueda M (2009): **Increase in E-selectin expression in umbilical vein endothelial cells by anticancer drugs and inhibition by cimetidine.** *Oncol Rep*, **22**(6):1293-1297
- Ke Q, Costa M (2006): **Hypoxia-inducible factor-1 (HIF-1).** *Mol Pharmacol*, **70**(5):1469-1480.
- Khan A (2012): **Detection and quantitation of forty eight cytokines, chemokines, growth factors and nine acute phase proteins in healthy human plasma, saliva and urine.** *J Proteomics*, **75**(15):4802-4819.
- Kilarski W (2005): **Mechanisms of Tissue Vascularization.** Uppsala, Sweden: Uppsala.
- Kim ES, Sohn YW, Moon A (2007): **TGF-beta-induced transcriptional activation of MMP-2 is mediated by activating transcription factor (ATF)2 in human breast epithelial cells.** *Cancer Lett*, **252**(1):147-156.
- Kim J, Ma T (2013): **Autocrine fibroblast growth factor 2-mediated interactions between human mesenchymal stem cells and the extracellular matrix under varying oxygen tension.** *J Cell Biochem*, **114**(3):716-727
- Kim YS, Min KS, Jeong DH, Jang JH, Kim HW, Kim EC (2010): **Effects of fibroblast growth factor-2 on the expression and regulation of chemokines in human dental pulp cells.** *J Endod*, **36**(11):1824-1830.
- Kitase Y, Yokozeki M, Fujihara S, Izawa T, Kuroda S, Tanimoto K, Moriyama K, Tanaka E (2009): **Analysis of gene expression profiles in human periodontal ligament cells under hypoxia: the protective effect of CC chemokine ligand 2 to oxygen shortage.** *Arch Oral Biol*, **54**(7):618-624
- Koch AE, Halloran MM, Haskell CJ, Shah MR, Polverini PJ (1995): **Angiogenesis mediated by soluble forms of E-selectin and vascular cell adhesion molecule-1.** *Nature*, **376**(6540):517-519.
- Kraiss LW, Alto NM, Dixon DA, McIntyre TM, Weyrich AS, Zimmerman GA (2003): **Fluid flow regulates E-selectin protein levels in human endothelial cells by inhibiting translation.** *J Vasc Surg*, **37**(1):161-168
- Kreil DP, Karp NA, Lilley KS (2004): **DNA microarray normalization methods can remove bias from differential protein expression analysis of 2D difference gel electrophoresis results.** *Bioinformatics*, **20**(13):2026-2034
- Krishnan V, Davidovitch Z (2006): **Cellular, molecular, and tissue-level reactions to orthodontic force.** *Am J Orthod Dentofacial Orthop*, **129**(4):469 e461-432.
- Krock BL, Skuli N, Simon MC (2011): **Hypoxia-induced angiogenesis: good and evil.** *Genes Cancer*, **2**(12):1117-1133
- Kroll J, Waltenberger J (1998): **VEGF-A induces expression of eNOS and iNOS in endothelial cells via VEGF receptor-2 (KDR).** *Biochem Biophys Res Commun*, **252**(3):743-746.

- Kuroda K, Sapadin A, Shoji T, Fleischmajer R, Lebwohl M (2001): **Altered expression of angiopoietins and Tie2 endothelium receptor in psoriasis.** *J Invest Dermatol*, **116**(5):713-720
- Kurz H, Burri PH, Djonov VG (2003): **Angiogenesis and vascular remodeling by intussusception: from form to function.** *News Physiol Sci*, **18**:65-70.
- Kuwabara K, Ogawa S, Matsumoto M, Koga S, Clauss M, Pinsky DJ, Lyn P, Leavy J, Witte L, Joseph-Silverstein J *et al* (1995): **Hypoxia-mediated induction of acidic/basic fibroblast growth factor and platelet-derived growth factor in mononuclear phagocytes stimulates growth of hypoxic endothelial cells.** *Proc Natl Acad Sci U S A*, **92**(10):4606-4610
- Kvinnsland S, Heyeraas K, Ofjord ES (1989): **Effect of experimental tooth movement on periodontal and pulpal blood flow.** *Eur J Orthod*, **11**(3):200-205.
- Landskron G, De la Fuente M, Thuwajit P, Thuwajit C, Hermoso MA (2014): **Chronic inflammation and cytokines in the tumor microenvironment.** *J Immunol Res*, **2014**:149185
- Lange C, Ehlken C, Martin G, Konzok K, Moscoso Del Prado J, Hansen LL, Agostini HT (2007): **Intravitreal injection of the heparin analog 5-amino-2-naphthalenesulfonate reduces retinal neovascularization in mice.** *Exp Eye Res*, **85**(3):323-327
- Laudanski P, Charkiewicz R, Kuzmicki M, Szamatowicz J, Swiatecka J, Mroczko B, Niklinski J (2014): **Profiling of selected angiogenesis-related genes in proliferative eutopic endometrium of women with endometriosis.** *Eur J Obstet Gynecol Reprod Biol*, **172**:85-92
- Lavine KJ, Kovacs A, Weinheimer C, Mann DL (2013): **Repetitive myocardial ischemia promotes coronary growth in the adult mammalian heart.** *J Am Heart Assoc*, **2**(5):e000343
- Lazzaretti DN, Bortoluzzi GS, Torres Fernandes LF, Rodriguez R, Grehs RA, Martins Hartmann MS (2014): **Histologic evaluation of human pulp tissue after orthodontic intrusion.** *J Endod*, **40**(10):1537-1540.
- Lebrin F, Deckers M, Bertolino P, Ten Dijke P (2005): **TGF-beta receptor function in the endothelium.** *Cardiovasc Res*, **65**(3):599-608.
- Lee HS, Kim CK (2011): **Effect of recombinant IL-10 on cultured fetal rat alveolar type II cells exposed to 65%-hyperoxia.** *Respir Res*, **12**:68.
- Lee YH, Kang YM, Heo MJ, Kim GE, Bhattarai G, Lee NH, Yu MK, Yi HK (2013): **The survival role of peroxisome proliferator-activated receptor gamma induces odontoblast differentiation against oxidative stress in human dental pulp cells.** *J Endod*, **39**(2):236-241.
- Lefevre G, Babchia N, Calipel A, Mouriaux F, Faussat AM, Mrzyk S, Mascarelli F (2009): **Activation of the FGF2/FGFR1 autocrine loop for cell proliferation and survival in uveal melanoma cells.** *Invest Ophthalmol Vis Sci*, **50**(3):1047-1057
- Lehner C, Gehwolf R, Tempfer H, Krizbai I, Hennig B, Bauer HC, Bauer H (2011): **Oxidative stress and blood-brain barrier dysfunction under particular consideration of matrix metalloproteinases.** *Antioxid Redox Signal*, **15**(5):1305-1323
- Lekanne Deprez RH, Fijnvandraat AC, Ruijter JM, Moorman AF (2002): **Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions.** *Anal Biochem*, **307**(1):63-69.

- Leone A, Mauro A, Spatola GF, Provenzano S, Caradonna C, Gerbino A, Buscemi M (2009): **MMP-2, MMP-9, and iNOS expression in human dental pulp subjected to orthodontic traction.** *Angle Orthod*, **79**(6):1119-1125
- Lewis CE, Pollard JW (2006): **Distinct role of macrophages in different tumor microenvironments.** *Cancer Res*, **66**(2):605-612
- Li A, Dubey S, Varney ML, Dave BJ, Singh RK (2003): **IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis.** *J Immunol*, **170**(6):3369-3376.
- Li F, Sonveaux P, Rabbani ZN, Liu S, Yan B, Huang Q, Vujaskovic Z, Dewhirst MW, Li CY (2007): **Regulation of HIF-1 α stability through S-nitrosylation.** *Mol Cell*, **26**(1):63-74
- Li J, Wei Y, Liu K, Yuan C, Tang Y, Quan Q, Chen P, Wang W, Hu H, Yang L (2010): **Synergistic effects of FGF-2 and PDGF-BB on angiogenesis and muscle regeneration in rabbit hindlimb ischemia model.** *Microvasc Res*, **80**(1):10-17
- Li J, Zhang YP, Kirsner RS (2003): **Angiogenesis in wound repair angiogenic growth factors and the extracellular matrix.** *Microsc Res Tech*, **60**(1):107-114
- Li L, Zhu YQ, Jiang L, Peng W, Ritchie HH (2011): **Hypoxia promotes mineralization of human dental pulp cells.** *J Endod*, **37**(6):799-802.
- Libby P, Warner SJ, Salomon RN, Birinyi LK (1988): **Production of platelet-derived growth factor-like mitogen by smooth-muscle cells from human atheroma.** *N Engl J Med*, **318**(23):1493-1498.
- Lin P, Polverini P, Dewhirst M, Shan S, Rao PS, Peters K (1997): **Inhibition of tumor angiogenesis using a soluble receptor establishes a role for Tie2 in pathologic vascular growth.** *J Clin Invest*, **100**(8):2072-2078
- Lin YJ, Markham NE, Balasubramaniam V, Tang JR, Maxey A, Kinsella JP, Abman SH (2005): **Inhaled nitric oxide enhances distal lung growth after exposure to hyperoxia in neonatal rats.** *Pediatr Res*, **58**(1):22-29
- Lindblom P, Gerhardt H, Liebner S, Abramsson A, Enge M, Hellstrom M, Backstrom G, Fredriksson S, Landegren U, Nystrom HC *et al* (2003): **Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall.** *Genes Dev*, **17**(15):1835-1840.
- <http://stores.biochem.uiowa.edu/Pages/ln2msds.htm>
- Liu J, Jin T, Chang S, Ritchie HH, Smith AJ, Clarkson BH (2007): **Matrix and TGF-beta-related gene expression during human dental pulp stem cell (DPSC) mineralization.** *In Vitro Cell Dev Biol Anim*, **43**(3-4):120-128.
- Liu L, Gao J, Yuan Y, Chang Q, Liao Y, Lu F (2013): **Hypoxia preconditioned human adipose derived mesenchymal stem cells enhance angiogenic potential via secretion of increased VEGF and bFGF.** *Cell Biol Int*, **37**(6):551-560.
- Livak KJ, Schmittgen TD (2001): **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.** *Methods*, **25**(4):402-408
- Lu S, Dong Z (2006): **Characterization of TGF-beta-regulated interleukin-8 expression in human prostate cancer cells.** *Prostate*, **66**(9):996-1004
- Luo JD, Chen AF (2005): **Nitric oxide: a newly discovered function on wound healing.** *Acta Pharmacol Sin*, **26**(3):259-264

- Makanya AN, Hlushchuk R, Djonov VG (2009): **Intussusceptive angiogenesis and its role in vascular morphogenesis, patterning, and remodeling.** *Angiogenesis*, **12**(2):113-123
- Manalo DJ, Rowan A, Lavoie T, Natarajan L, Kelly BD, Ye SQ, Garcia JG, Semenza GL (2005): **Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1.** *Blood*, **105**(2):659-669
- Martin V, Liu D, Fueyo J, Gomez-Manzano C (2008): **Tie2 a journey from normal angiogenesis to cancer and beyond.** *Histol Histopathol*, **23**(6):773-780.
- Matsushita K, Motani R, Sakuta T, Yamaguchi N, Koga T, Matsuo K, Nagaoka S, Abeyama K, Maruyama I, Torii M (2000): **The role of vascular endothelial growth factor in human dental pulp cells: induction of chemotaxis, proliferation, and differentiation and activation of the AP-1-dependent signaling pathway.** *J Dent Res*, **79**(8):1596-1603.
- Mavragani M, Amundsen OC, Selliseth NJ, Brudvik P, Selvig KA (2004): **Early root alterations after orthodontic force application studied by light and scanning electron microscopy.** *Eur J Orthod*, **26**(2):119-128.
- Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ (1999): **The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis.** *Nature*, **399**(6733):271-275.
- McCluskie K, Birrell MA, Wong S, Belvisi MG (2004): **Nitric oxide as a noninvasive biomarker of lipopolysaccharide-induced airway inflammation: possible role in lung neutrophilia.** *J Pharmacol Exp Ther*, **311**(2):625-633.
- McLachlan JL, Smith AJ, Bujalska IJ, Cooper PR (2005): **Gene expression profiling of pulpal tissue reveals the molecular complexity of dental caries.** *Biochim Biophys Acta*, **1741**(3):271-281.
- McMahon S, Charbonneau M, Grandmont S, Richard DE, Dubois CM (2006): **Transforming growth factor beta1 induces hypoxia-inducible factor-1 stabilization through selective inhibition of PHD2 expression.** *J Biol Chem*, **281**(34):24171-24181.
- Medeiros M, Sharma VK, Ding R, Yamaji K, Li B, Muthukumar T, Valderde-Rosas S, Hernandez AM, Munoz R, Suthanthiran M (2003): **Optimization of RNA yield, purity and mRNA copy number by treatment of urine cell pellets with RNAlater.** *J Immunol Methods*, **279**(1-2):135-142.
- Meeran NA (2013): **Cellular response within the periodontal ligament on application of orthodontic forces.** *J Indian Soc Periodontol*, **17**(1):16-20
- Meyer-Ingold W, Eichner W (1995): **Platelet-derived growth factor.** *Cell Biol Int*, **19**(5):389-398
- Millauer B, Witzigmann-Voos S, Schnurch H, Martinez R, Moller NP, Risau W, Ullrich A (1993): **High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis.** *Cell*, **72**(6):835-846.
- Miyagawa A, Chiba M, Hayashi H, Igarashi K (2009): **Compressive force induces VEGF production in periodontal tissues.** *J Dent Res*, **88**(8):752-756

- Mizuno M, Katano H, Otabe K, Komori K, Matsumoto Y, Fujii S, Ozeki N, Tsuji K, Koga H, Muneta T *et al* (2015): **Platelet-derived growth factor (PDGF)-AA/AB in human serum are potential indicators of the proliferative capacity of human synovial mesenchymal stem cells.** *Stem Cell Res Ther*, **6**:243.
- Mocchetti I, Rabin SJ, Colangelo AM, Whittemore SR, Wrathall JR (1996): **Increased basic fibroblast growth factor expression following contusive spinal cord injury.** *Exp Neurol*, **141**(1):154-164
- Moeller BJ, Cao Y, Vujaskovic Z, Li CY, Haroon ZA, Dewhirst MW (2004): **The relationship between hypoxia and angiogenesis.** *Semin Radiat Oncol*, **14**(3):215-221.
- Moen I, Oyan AM, Kalland KH, Tronstad KJ, Akslen LA, Chekenya M, Sakariassen PO, Reed RK, Stuhr LE (2009): **Hyperoxic treatment induces mesenchymal-to-epithelial transition in a rat adenocarcinoma model.** *PLoS One*, **4**(7):e6381
- Moffett J, Kratz E, Myers J, Stachowiak EK, Florkiewicz RZ, Stachowiak MK (1998): **Transcriptional regulation of fibroblast growth factor-2 expression in human astrocytes: implications for cell plasticity.** *Mol Biol Cell*, **9**(8):2269-2285
- Mostafa YA, Iskander KG, El-Mangoury NH (1991): **Iatrogenic pulpal reactions to orthodontic extrusion.** *Am J Orthod Dentofacial Orthop*, **99**(1):30-34.
- Mottet D, Bellahcene A, Pirotte S, Waltregny D, Deroanne C, Lamour V, Lidereau R, Castronovo V (2007): **Histone deacetylase 7 silencing alters endothelial cell migration, a key step in angiogenesis.** *Circ Res*, **101**(12):1237-1246
- Murakami M (2012): **Signaling required for blood vessel maintenance: molecular basis and pathological manifestations.** *Int J Vasc Med*, **2012**:293641
- Nagata JY, Gomes BP, Rocha Lima TF, Murakami LS, de Faria DE, Campos GR, de Souza-Filho FJ, Soares Ade J (2014): **Traumatized immature teeth treated with 2 protocols of pulp revascularization.** *J Endod*, **40**(5):606-612
- Nakagawa T, Li JH, Garcia G, Mu W, Piek E, Bottinger EP, Chen Y, Zhu HJ, Kang DH, Schreiner GF *et al* (2004): **TGF-beta induces proangiogenic and antiangiogenic factors via parallel but distinct Smad pathways.** *Kidney Int*, **66**(2):605-613
- Nakamura Y, Noda K, Shimoda S, Oikawa T, Arai C, Nomura Y, Kawasaki K (2008): **Time-lapse observation of rat periodontal ligament during function and tooth movement, using microcomputed tomography.** *Eur J Orthod*, **30**(3):320-326
- Nakanishi H, Seki Y, Kohno T, Muramoto T, Toda K, Soma K (2004): **Changes in response properties of periodontal mechanoreceptors after experimental orthodontic tooth movement in rats.** *Angle Orthod*, **74**(1):93-99.
- Neve A, Cantatore FP, Maruotti N, Corrado A, Ribatti D (2014): **Extracellular matrix modulates angiogenesis in physiological and pathological conditions.** *Biomed Res Int*, **2014**:756078
- Niland S, Eble JA (2012): **Integrin-mediated cell-matrix interaction in physiological and pathological blood vessel formation.** *J Oncol*, **2012**:125278

- Nilsson I, Shibuya M, Wennstrom S (2004): **Differential activation of vascular genes by hypoxia in primary endothelial cells.** *Exp Cell Res*, **299**(2):476-485.
- Ning Y, Manegold PC, Hong YK, Zhang W, Pohl A, Lurje G, Winder T, Yang D, Labonte MJ, Wilson PM *et al* (2011): **Interleukin-8 is associated with proliferation, migration, angiogenesis and chemosensitivity *in vitro* and *in vivo* in colon cancer cell line models.** *International Journal of Cancer*, **128**:2038-2049.
- Nishishita T, Lin PC (2004): **Angiopoietin 1, PDGF-B, and TGF-beta gene regulation in endothelial cell and smooth muscle cell interaction.** *J Cell Biochem*, **91**(3):584-593
- Nishiwaki Y, Yoshida M, Iwaguro H, Masuda H, Nitta N, Asahara T, Isobe M (2007): **Endothelial E-selectin potentiates neovascularization via endothelial progenitor cell-dependent and -independent mechanisms.** *Arterioscler Thromb Vasc Biol*, **27**(3):512-518
- Nixon RG, Wener MH, Smith KM, Parson RE, Strobel SA, Brawer MK (1997): **Biological variation of prostate specific antigen levels in serum: an evaluation of day-to-day physiological fluctuations in a well-defined cohort of 24 patients.** *J Urol*, **157**(6):2183-2190.
- Noda K, Nakamura Y, Kogure K, Nomura Y (2009): **Morphological changes in the rat periodontal ligament and its vascularity after experimental tooth movement using superelastic forces.** *Eur J Orthod*, **31**(1):37-45
- Nolan T, Hands RE, Bustin SA (2006): **Quantification of mRNA using real-time RT-PCR.** *Nat Protoc*, **1**(3):1559-1582.
- Ohlsson R, Falck P, Hellstrom M, Lindahl P, Bostrom H, Franklin G, Ahrlund-Richter L, Pollard J, Soriano P, Betsholtz C (1999): **PDGFB regulates the development of the labyrinthine layer of the mouse fetal placenta.** *Dev Biol*, **212**(1):124-136
- Okamura K, Kobayashi I, Matsuo K, Taniguchi K, Ishibashi Y, Izumi T, Sakai H (1994): **Ultrastructure of the neuromuscular junction of vasomotor nerves in the microvasculature of human dental pulp.** *Arch Oral Biol*, **39**(3):171-176.
- Oppenheim A (2007): **Tissue changes, particularly of the bone, incident to tooth movement.** *Eur J Orthod*, **29**((suppl 1)):13.
- Ozaki H, Yu AY, Della N, Ozaki K, Luna JD, Yamada H, Hackett SF, Okamoto N, Zack DJ, Semenza GL *et al* (1999): **Hypoxia inducible factor-1alpha is increased in ischemic retina: temporal and spatial correlation with VEGF expression.** *Invest Ophthalmol Vis Sci*, **40**(1):182-189
- Ozkan A, Bicer A, Avsar T, Seker A, Toktas ZO, Bozkurt SU, Basak AN, Kilic T (2012): **Temporal expression analysis of angiogenesis-related genes in brain development.** *Vasc Cell*, **4**(1):16
- Paakkonen V, Ohlmeier S, Bergmann U, Larmas M, Salo T, Tjaderhane L (2005): **Analysis of gene and protein expression in healthy and carious tooth pulp with cDNA microarray and two-dimensional gel electrophoresis.** *Eur J Oral Sci*, **113**(5):369-379.
- Paakkonen V, Tjaderhane L (2010): **High-throughput gene and protein expression analysis in pulp biologic research review.** *J Endod*, **36**(2):179-189.

- Palomero J, Vegliante MC, Rodriguez ML, Eguileor A, Castellano G, Planas-Rigol E, Jares P, Ribera-Cortada I, Cid MC, Campo E *et al* (2014): **SOX11 promotes tumor angiogenesis through transcriptional regulation of PDGFA in mantle cell lymphoma.** *Blood*, **124**(14):2235-2247.
- Park MY, Jeong YJ, Kang GC, Kim MH, Kim SH, Chung HJ, Jung JY, Kim WJ (2014): **Nitric oxide-induced apoptosis of human dental pulp cells is mediated by the mitochondria-dependent pathway.** *Korean J Physiol Pharmacol*, **18**(1):25-32.
- Paternostro C, David E, Novo E, Parola M (2010): **Hypoxia, angiogenesis and liver fibrogenesis in the progression of chronic liver diseases.** *World J Gastroenterol*, **16**(3):281-288
- Peirson SN, Butler JN, Foster RG (2003): **Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis.** *Nucleic Acids Res*, **31**(14):e73.
- Peplow PV, Baxter GD (2012): **Gene expression and release of growth factors during delayed wound healing: a review of studies in diabetic animals and possible combined laser phototherapy and growth factor treatment to enhance healing.** *Photomed Laser Surg*, **30**(11):617-636.
- Perez S, Royo LJ, Astudillo A, Escudero D, Alvarez F, Rodriguez A, Gomez E, Otero J (2007): **Identifying the most suitable endogenous control for determining gene expression in hearts from organ donors.** *BMC Mol Biol*, **8**:114.
- Pfaffl MW, Horgan GW, Dempfle L (2002): **Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR.** *Nucleic Acids Res*, **30**(9):e36
- Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004): **Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations.** *Biotechnol Lett*, **26**(6):509-515.
- Pfaffl MW (2001): **A new mathematical model for relative quantification in real-time RT-PCR.** *Nucleic Acids Res*, **29**(9):e45
- Pham I, Uchida T, Planes C, Ware LB, Kaner R, Matthay MA, Clerici C (2002): **Hypoxia upregulates VEGF expression in alveolar epithelial cells in vitro and in vivo.** *Am J Physiol Lung Cell Mol Physiol*, **283**(5):L1133-1142.
- Phng LK, Stanchi F, Gerhardt H (2013): **Filopodia are dispensable for endothelial tip cell guidance.** *Development*, **140**(19):4031-4040.
- Pierce GF, Tarpley JE, Tseng J, Bready J, Chang D, Kenney WC, Rudolph R, Robson MC, Vande Berg J, Reid P *et al* (1995): **Detection of platelet-derived growth factor (PDGF)-AA in actively healing human wounds treated with recombinant PDGF-BB and absence of PDGF in chronic nonhealing wounds.** *J Clin Invest*, **96**(3):1336-1350
- Pietras K, Ostman A, Sjoquist M, Buchdunger E, Reed RK, Heldin CH, Rubin K (2001): **Inhibition of platelet-derived growth factor receptors reduces interstitial hypertension and increases transcapillary transport in tumors.** *Cancer Res*, **61**(7):2929-2934.
- Pilon JJ, Kuijpers-Jagtman AM, Maltha JC (1996): **Magnitude of orthodontic forces and rate of bodily tooth movement. An experimental study.** *Am J Orthod Dentofacial Orthop*, **110**(1):16-23

- Pisciotta A, Carnevale G, Meloni S, Riccio M, De Biasi S, Gibellini L, Ferrari A, Bruzzesi G, De Pol A (2015): **Human dental pulp stem cells (hDPSCs): isolation, enrichment and comparative differentiation of two sub-populations.** *BMC Dev Biol*, **15**:14.
- Polotsky VY, Li J, Punjabi NM, Rubin AE, Smith PL, Schwartz AR, O'Donnell CP (2003): **Intermittent hypoxia increases insulin resistance in genetically obese mice.** *J Physiol*, **552**(Pt 1):253-264.
- Pombo-Suarez M, Calaza M, Gomez-Reino JJ, Gonzalez A (2008): **Reference genes for normalization of gene expression studies in human osteoarthritic articular cartilage.** *BMC Mol Biol*, **9**:17
- Porter KM, Kang BY, Adesina SE, Murphy TC, Hart CM, Sutliff RL (2014): **Chronic hypoxia promotes pulmonary artery endothelial cell proliferation through H2O2-induced 5-lipoxygenase.** *PLoS One*, **9**(6):e98532
- Powell FL, Garcia N (2000): **Physiological effects of intermittent hypoxia.** *High Alt Med Biol*, **1**(2):125-136
- Presta M, Andres G, Leali D, Dell'Era P, Ronca R (2009): **Inflammatory cells and chemokines sustain FGF2-induced angiogenesis.** *Eur Cytokine Netw*, **20**(2):39-50
- Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M (2005): **Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis.** *Cytokine Growth Factor Rev*, **16**(2):159-178
- Provenza DV (1958): **The blood vascular supply of the dental pulp with emphasis on capillary circulation.** *Circ Res*, **6**(2):213-218.
- Pugh CW, Ratcliffe PJ (2003): **Regulation of angiogenesis by hypoxia: role of the HIF system.** *Nat Med*, **9**(6):677-684
- Rahbari NN, Schmidt T, Falk CS, Hinz U, Herber M, Bork U, Buchler MW, Weitz J, Koch M (2011): **Expression and prognostic value of circulating angiogenic cytokines in pancreatic cancer.** *BMC Cancer*, **11**:286
- Rahman MM, Matsuoka K, Takeshita S, Ikeda K (2015): **Secretion of PDGF isoforms during osteoclastogenesis and its modulation by anti-osteoclast drugs.** *Biochem Biophys Res Commun*, **462**(2):159-164.
- Raja R, Kale S, Thorat D, Soundararajan G, Lohite K, Mane A, Karnik S, Kundu GC (2014): **Hypoxia-driven osteopontin contributes to breast tumor growth through modulation of HIF1alpha-mediated VEGF-dependent angiogenesis.** *Oncogene*, **33**(16):2053-2064.
- Ramazanzadeh BA, Sahhafian AA, Mohtasham N, Hassanzadeh N, Jahanbin A, Shakeri MT (2009): **Histological changes in human dental pulp following application of intrusive and extrusive orthodontic forces.** *J Oral Sci*, **51**(1):109-115
- Rasheed S, Yan JS, Hussain A, Lai B (2009): **Proteomic characterization of HIV-modulated membrane receptors, kinases and signaling proteins involved in novel angiogenic pathways.** *J Transl Med*, **7**:75
- Reed RK, Rubin K (2010): **Transcapillary exchange: role and importance of the interstitial fluid pressure and the extracellular matrix.** *Cardiovasc Res*, **87**(2):211-217.
- Ren Y, Maltha JC, Stokroos I, Liem RS, Kuijpers-Jagtman AM (2008): **Effect of duration of force application on blood vessels in young and adult rats.** *Am J Orthod Dentofacial Orthop*, **133**(5):752-757
- <http://rest-2008.gene-quantification.info/>

- Resuehr D, Spiess AN (2003): **A real-time polymerase chain reaction-based evaluation of cDNA synthesis priming methods.** *Anal Biochem*, **322**(2):287-291
- Revillion F, Pawlowski V, Hornez L, Peyrat JP (2000): **Glyceraldehyde-3-phosphate dehydrogenase gene expression in human breast cancer.** *Eur J Cancer*, **36**(8):1038-1042.
- Rhinn H, Marchand-Leroux C, Croci N, Plotkine M, Scherman D, Escriou V (2008): **Housekeeping while brain's storming Validation of normalizing factors for gene expression studies in a murine model of traumatic brain injury.** *BMC Mol Biol*, **9**:62.
- Riabov V, Gudima A, Wang N, Mickley A, Orekhov A, Kzhyshkowska J (2014): **Role of tumor associated macrophages in tumor angiogenesis and lymphangiogenesis.** *Front Physiol*, **5**:75
- Ricos C, Perich C, Minchinela J, Alvarez V, Simon M, Biosca C, Domenech M, Fernandez P, Jimenez CV, Garcia-Lario JV *et al* (2009): **Application of Biological Variation - A Review.** *Biochemia Medica*, **19**(3):250-259.
- Risau W (1997): **Mechanisms of angiogenesis.** *Nature*, **386**(6626):671-674
- Risau W (1996): **What, if anything, is an angiogenic factor?** *Cancer Metastasis Rev*, **15**(2):149-151
- Rivera LB, Brekken RA (2011): **SPARC promotes pericyte recruitment via inhibition of endoglin-dependent TGF-beta1 activity.** *J Cell Biol*, **193**(7):1305-1319.
- <http://www.chem.agilent.com/rin/rinsearch.aspx>.
- Roberts-Clark DJ, Smith AJ (2000): **Angiogenic growth factors in human dentine matrix.** *Arch Oral Biol*, **45**(11):1013-1016.
- Rodd HD, Boissonade FM (2005): **Vascular status in human primary and permanent teeth in health and disease.** *Eur J Oral Sci*, **113**(2):128-134.
- Romer P, Wolf M, Fanghanel J, Reicheneder C, Proff P (2014): **Cellular response to orthodontically-induced short-term hypoxia in dental pulp cells.** *Cell Tissue Res*, **355**(1):173-180.
- Ruch RJ, Crist KA, Klaunig JE (1989): **Effects of culture duration on hydrogen peroxide-induced hepatocyte toxicity.** *Toxicol Appl Pharmacol*, **100**(3):451-464.
- Ruijter JM, Pfaffl MW, Zhao S, Spiess AN, Boggy G, Blom J, Rutledge RG, Sisti D, Lievens A, De Preter K *et al* (2013): **Evaluation of qPCR curve analysis methods for reliable biomarker discovery: bias, resolution, precision, and implications.** *Methods*, **59**(1):32-46.
- Rundhaug JE (2005): **Matrix metalloproteinases and angiogenesis.** *J Cell Mol Med*, **9**(2):267-285.
- Saalbach A, Wetzel A, Hausteil UF, Sticherling M, Simon JC, Anderegg U (2005): **Interaction of human Thy-1 (CD 90) with the integrin alphavbeta3 (CD51/CD61): an important mechanism mediating melanoma cell adhesion to activated endothelium.** *Oncogene*, **24**(29):4710-4720.
- Saikumar P, Dong Z, Weinberg JM, Venkatachalam MA (1998): **Mechanisms of cell death in hypoxia/reoxygenation injury.** *Oncogene*, **17**(25):3341-3349.
- Sakdee JB, White RR, Pagonis TC, Hauschka PV (2009): **Hypoxia-amplified proliferation of human dental pulp cells.** *J Endod*, **35**(6):818-823.
- Saliba AE, Westermann AJ, Gorski SA, Vogel J (2014): **Single-cell RNA-seq: advances and future challenges.** *Nucleic Acids Res*, **42**(14):8845-8860.

- Sambrook J, Fritsch EF, Maniatis T (1989): **Molecular cloning : a laboratory manual**, 2nd ed. edn. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.
- Sanders R, Mason DJ, Foy CA, Huggett JF (2013): **Evaluation of digital PCR for absolute RNA quantification**. *PLoS One*, **8**(9):e75296.
- Sankar S, Mahooti-Brooks N, Bensen L, McCarthy TL, Centrella M, Madri JA (1996): **Modulation of transforming growth factor beta receptor levels on microvascular endothelial cells during in vitro angiogenesis**. *J Clin Invest*, **97**(6):1436-1446
- Sano Y, Ikawa M, Sugawara J, Horiuchi H, Mitani H (2002): **The effect of continuous intrusive force on human pulpal blood flow**. *Eur J Orthod*, **24**(2):159-166
- Santamaria M, Jr., Milagres D, Stuaní AS, Stuaní MB, Ruellas AC (2006): **Initial changes in pulpal microvasculature during orthodontic tooth movement: a stereological study**. *Eur J Orthod*, **28**(3):217-220.
- Schmalz G (2014): **Pulp Reactions to Dental Materials**. In: *The Dental Pulp: Biology, Pathology and Regenerative Therapies*. Edited by Goldberg M. Heidelberg, New York, Dordrecht, London: Springer-Verlag Berlin Heidelberg; : 169-184.
- Schmittgen TD, Livak KJ (2008): **Analyzing real-time PCR data by the comparative C(T) method**. *Nat Protoc*, **3**(6):1101-1108
- Schmittgen TD, Zakrajsek BA (2000): **Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR**. *J Biochem Biophys Methods*, **46**(1-2):69-81.
- Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M, Ragg T (2006): **The RIN: an RNA integrity number for assigning integrity values to RNA measurements**. *BMC Mol Biol*, **7**:3.
- Selvey S, Thompson EW, Matthaei K, Lea RA, Irving MG, Griffiths LR (2001): **Beta-actin--an unsuitable internal control for RT-PCR**. *Mol Cell Probes*, **15**(5):307-311.
- Semenza GL (2010): **Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics**. *Oncogene*, **29**(5):625-634.
- Semenza GL (2001): **Regulation of hypoxia-induced angiogenesis: a chaperone escorts VEGF to the dance**. *J Clin Invest*, **108**(1):39-40.
- Semenza GL (2003): **Targeting HIF-1 for cancer therapy**. *Nat Rev Cancer*, **3**(10):721-732.
- Serini G, Valdembri D, Bussolino F (2006): **Integrins and angiogenesis: a sticky business**. *Exp Cell Res*, **312**(5):651-658.
- Shattil SJ, Newman PJ (2004): **Integrins dynamic scaffolds for adhesion and signaling in platelets**. *Blood*, **104**(6):1606-1615.
- Shih AH, Holland EC (2006): **Platelet-derived growth factor (PDGF) and glial tumorigenesis**. *Cancer Lett*, **232**(2):139-147
- Shikada Y, Yonemitsu Y, Koga T, Onimaru M, Nakano T, Okano S, Sata S, Nakagawa K, Yoshino I, Maehara Y *et al* (2005): **Platelet-derived growth factor-AA is an essential and autocrine regulator of vascular endothelial growth factor expression in non-small cell lung carcinomas**. *Cancer Res*, **65**(16):7241-7248

- Shimabukuro Y, Ueda M, Ichikawa T, Terashi Y, Yamada S, Kusumoto Y, Takedachi M, Terakura M, Kohya A, Hashikawa T *et al* (2005): **Fibroblast growth factor-2 stimulates hyaluronan production by human dental pulp cells.** *J Endod*, **31**(11):805-808.
- Shimabukuro Y, Ueda M, Ozasa M, Anzai J, Takedachi M, Yanagita M, Ito M, Hashikawa T, Yamada S, Murakami S (2009): **Fibroblast growth factor-2 regulates the cell function of human dental pulp cells.** *J Endod*, **35**(11):1529-1535.
- Short RHD (1950): **Alveolar Epithelium in Relation to Growth of the Lung.** *Philosophical Transactions of the Royal Society of London Series B, Biological Sciences*, **235**(622):51
- Shweiki D, Itin A, Soffer D, Keshet E (1992): **Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis.** *Nature*, **359**(6398):843-845.
- Silletti S, Kessler T, Goldberg J, Boger DL, Cheresch DA (2001): **Disruption of matrix metalloproteinase 2 binding to integrin alpha vbeta 3 by an organic molecule inhibits angiogenesis and tumor growth in vivo.** *Proc Natl Acad Sci U S A*, **98**(1):119-124
- Silpanisong J, Pearce WJ (2013): **Vasotrophic regulation of age-dependent hypoxic cerebrovascular remodeling.** *Curr Vasc Pharmacol*, **11**(5):544-563
- Silva AC, Faria MR, Fontes A, Campos MS, Cavalcanti BN (2009): **Interleukin-1 beta and interleukin-8 in healthy and inflamed dental pulps.** *J Appl Oral Sci*, **17**(5):527-532.
- Singh H, Tahir TA, Alawo DO, Issa E, Brindle NP (2011): **Molecular control of angiopoietin signalling.** *Biochem Soc Trans*, **39**(6):1592-1596
- Singh K, Balligand JL, Fischer TA, Smith TW, Kelly RA (1996): **Regulation of cytokine-inducible nitric oxide synthase in cardiac myocytes and microvascular endothelial cells. Role of extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2) and STAT1 alpha.** *J Biol Chem*, **271**(2):1111-1117.
- Sirover MA (1997): **Role of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in normal cell function and in cell pathology.** *J Cell Biochem*, **66**(2):133-140.
- Sloan AJ, Couble ML, Bleicher F, Magloire H, Smith AJ, Farges JC (2001): **Expression of TGF-beta receptors I and II in the human dental pulp by in situ hybridization.** *Adv Dent Res* **15**:63-67.
- Smith AJ (2002): **Pulpal responses to caries and dental repair.** *Caries Res*, **36**(4):223-232.
- Smith SJ, Cooper GR, Myers GL, Sampson EJ (1993): **Biological variability in concentrations of serum lipids: sources of variation among results from published studies and composite predicted values.** *Clin Chem*, **39**(6):1012-1022.
- Smith WB, Noack L, Khew-Goodall Y, Isenmann S, Vadas MA, Gamble JR (1996): **Transforming growth factor-beta 1 inhibits the production of IL-8 and the transmigration of neutrophils through activated endothelium.** *J Immunol*, **157**(1):360-368.
- Solanas M, Moral R, Escrich E (2001): **Unsuitability of using ribosomal RNA as loading control for Northern blot analyses related to the imbalance between messenger and ribosomal RNA content in rat mammary tumors.** *Anal Biochem*, **288**(1):99-102.

- Song JW, Bazou D, Munn LL (2012): **Anastomosis of endothelial sprouts forms new vessels in a tissue analogue of angiogenesis.** *Integr Biol (Camb)*, **4**(8):857-862.
- Spanakis E (1993): **Problems related to the interpretation of autoradiographic data on gene expression using common constitutive transcripts as controls.** *Nucleic Acids Res*, **21**(16):3809-3819.
- Spielman RS, Bastone LA, Burdick JT, Morley M, Ewens WJ, Cheung VG (2007): **Common genetic variants account for differences in gene expression among ethnic groups.** *Nat Genet*, **39**(2):226-231
- Spina A, Montella R, Liccardo D, De Rosa A, Laino L, Mitsiadis TA, La Noce M (2016): **NZ-GMP Approved Serum Improve hDPSC Osteogenic Commitment and Increase Angiogenic Factor Expression.** *Front Physiol*, **7**:354.
- Stachowiak MK, Moffett J, Maher P, Tucholski J, Stachowiak EK (1997): **Growth factor regulation of cell growth and proliferation in the nervous system. A new intracrine nuclear mechanism.** *Mol Neurobiol*, **15**(3):257-283
- Stahlberg A, Kubista M, Pfaffl M (2004): **Comparison of reverse transcriptases in gene expression analysis.** *Clin Chem*, **50**(9):1678-1680.
- Staquet MJ, Couble ML, Romeas A, Connolly M, Magloire H, Hynes RO, Clezardin P, Bleicher F, Farges JC (2006): **Expression and localisation of alphav integrins in human odontoblasts.** *Cell Tissue Res*, **323**(3):457-463.
- Stenvik A, Mjor IA (1970): **Pulp and dentine reactions to experimental tooth intrusion. A histologic study of the initial changes.** *Am J Orthod*, **57**(4):370-385.
- Stern-Straeter J, Bonaterra GA, Hormann K, Kinscherf R, Goessler UR (2009): **Identification of valid reference genes during the differentiation of human myoblasts.** *BMC Mol Biol*, **10**(1):66.
- Stockmann C, Schadendorf D, Klose R, Helfrich I (2014): **The impact of the immune system on tumor: angiogenesis and vascular remodeling.** *Front Oncol*, **4**:69
- Styp-Rekowska B, Hlushchuk R, Pries AR, Djonov V (2011): **Intussusceptive angiogenesis: pillars against the blood flow.** *Acta Physiol (Oxf)*, **202**(3):213-223.
- Subay RK, Kaya H, Tarim B, Subay A, Cox CF (2001): **Response of human pulpal tissue to orthodontic extrusive applications.** *J Endod*, **27**(8):508-511
- Suchanek J, Klepova TS, Kapitan M, Soukup T (2013): **The effect of fetal calf serum on human dental pulp stem cells.** *Acta Medica (Hradec Kralove)*, **56**(4):142-149.
- Sukhotnik I, Coran AG, Greenblatt R, Brod V, Mogilner J, Shiloni E, Shaoul R, Bitterman H (2008): **Effect of 100% oxygen on E-selectin expression, recruitment of neutrophils and enterocyte apoptosis following intestinal ischemia-reperfusion in a rat.** *Pediatr Surg Int*, **24**(1):29-35
- Sukhotnik I, Mogilner JG, Pollak Y, Blumenfeld S, Bejar J, Coran AG (2012): **PDGF-alpha stimulates intestinal epithelial cell turnover after massive small bowel resection in a rat.** *Am J Physiol Gastrointest Liver Physiol*, **302**(11):G1274-1281
- Sumpio BE, Riley JT, Dardik A (2002): **Cells in focus: endothelial cell.** *Int J Biochem Cell Biol*, **34**(12):1508-1512.
- Suzuki T, Higgins PJ, Crawford DR (2000): **Control selection for RNA quantitation.** *Biotechniques*, **29**(2):332-337.

- Takagi H, King GL, Ferrara N, Aiello LP (1996): **Hypoxia regulates vascular endothelial growth factor receptor KDR/Flk gene expression through adenosine A2 receptors in retinal capillary endothelial cells.** *Invest Ophthalmol Vis Sci*, **37**(7):1311-1321.
- Talwar T, Srivastava MV (2014): **Role of vascular endothelial growth factor and other growth factors in post-stroke recovery.** *Ann Indian Acad Neurol*, **17**(1):1-6
- Tatullo M, Marrelli M, Shakesheff KM, White LJ (2015): **Dental pulp stem cells: function, isolation and applications in regenerative medicine.** *J Tissue Eng Regen Med*, **9**(11):1205-1216.
- Taube A, Schlich R, Sell H, Eckardt K, Eckel J (2012): **Inflammation and metabolic dysfunction: links to cardiovascular diseases.** *Am J Physiol Heart Circ Physiol*, **302**(11):H2148-2165.
- Tawab A, Fan Y, Read EJ, Kurlander RJ (2009): **Effect of ex vivo culture duration on phenotype and cytokine production by mature dendritic cells derived from peripheral blood monocytes.** *Transfusion*, **49**(3):536-547.
- ten Dijke P, Arthur HM (2007): **Extracellular control of TGFbeta signalling in vascular development and disease.** *Nat Rev Mol Cell Biol*, **8**(11):857-869.
- Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, Bohlen P (1992): **Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor.** *Biochem Biophys Res Commun*, **187**(3):1579-1586.
- Tete S, Mastrangelo F, Scioletti AP, Tranasi M, Raicu F, Paolantonio M, Stuppia L, Vinci R, Gherlone E, Ciampoli C *et al* (2008): **Microarray expression profiling of human dental pulp from single subject.** *Clin Invest Med*, **31**(2):E55-61.
- Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E (1999): **Housekeeping genes as internal standards: use and limits.** *J Biotechnol*, **75**(2-3):291-295.
- Thomas M, Augustin HG (2009): **The role of the Angiopoietins in vascular morphogenesis.** *Angiogenesis*, **12**(2):125-137.
- Ting L, Cowley MJ, Hoon SL, Guilhaus M, Raftery MJ, Cavicchioli R (2009): **Normalization and statistical analysis of quantitative proteomics data generated by metabolic labeling.** *Mol Cell Proteomics*, **8**(10):2227-2242
- Toegel S, Huang W, Piana C, Unger FM, Wirth M, Goldring MB, Gabor F, Viernstein H (2007): **Selection of reliable reference genes for qPCR studies on chondroprotective action.** *BMC Mol Biol*, **8**:13
- Toffoli S, Roegiers A, Feron O, Van Steenbrugge M, Ninane N, Raes M, Michiels C (2009): **Intermittent hypoxia is an angiogenic inducer for endothelial cells: role of HIF-1.** *Angiogenesis*, **12**(1):47-67.
- Toyono T, Nakashima M, Kuhara S, Akamine A (1997): **Expression of TGF-beta superfamily receptors in dental pulp.** *J Dent Res*, **76**(9):1555-1560.
- Tran-Hung L, Laurent P, Camps J, About I (2008): **Quantification of angiogenic growth factors released by human dental cells after injury.** *Arch Oral Biol*, **53**(1):9-13
- Tran-Hung L, Mathieu S, About I (2006): **Role of human pulp fibroblasts in angiogenesis.** *J Dent Res*, **85**(9):819-823
- Tressel SL, Huang RP, Tomsen N, Jo H (2007): **Laminar shear inhibits tubule formation and migration of endothelial cells by an angiopoietin-2 dependent mechanism.** *Arterioscler Thromb Vasc Biol*, **27**(10):2150-2156.

- Tricarico C, Pinzani P, Bianchi S, Paglierani M, Distante V, Pazzagli M, Bustin SA, Orlando C (2002): **Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies.** *Anal Biochem*, **309**(2):293-300.
- Tripuwabhrut P, Brudvik P, Fristad I, Rethnam S (2010): **Experimental orthodontic tooth movement and extensive root resorption: periodontal and pulpal changes.** *Eur J Oral Sci*, **118**(6):596-603.
- Tsuzuki T, Okada H, Cho H, Shimoi K, Miyashiro H, Yasuda K, Kanzaki H (2013): **Divergent regulation of angiopoietin-1, angiopoietin-2, and vascular endothelial growth factor by hypoxia and female sex steroids in human endometrial stromal cells.** *Eur J Obstet Gynecol Reprod Biol*, **168**(1):95-101.
- Uebelhoer M, Natynki M, Kangas J, Mendola A, Nguyen HL, Soblet J, Godfraind C, Boon LM, Eklund L, Limaye N *et al* (2013): **Venous malformation-causative TIE2 mutations mediate an AKT-dependent decrease in PDGFB.** *Hum Mol Genet*, **22**(17):3438-3448
- Ueda L, Matsushima K (2001): **Stimulation of plasminogen activator activity and matrix metalloproteinases of human dental pulp-derived cells by tumor necrosis factor-alpha.** *J Endod*, **27**(3):175-179.
- Uematsu S, Mogi M, Deguchi T (1996): **Increase of transforming growth factor-beta 1 in gingival crevicular fluid during human orthodontic tooth movement.** *Arch Oral Biol*, **41**(11):1091-1095.
- Vacca A, Ria R, Semeraro F, Merchionne F, Coluccia M, Boccarelli A, Scavelli C, Nico B, Gernone A, Battelli F *et al* (2003): **Endothelial cells in the bone marrow of patients with multiple myeloma.** *Blood*, **102**(9):3340-3348
- Valente V, Teixeira SA, Neder L, Okamoto OK, Oba-Shinjo SM, Marie SK, Scrideli CA, Paco-Larson ML, Carlotti CG, Jr. (2009): **Selection of suitable housekeeping genes for expression analysis in glioblastoma using quantitative RT-PCR.** *BMC Mol Biol*, **10**:17.
- Valter K, Maslim J, Bowers F, Stone J (1998): **Photoreceptor dystrophy in the RCS rat: roles of oxygen, debris, and bFGF.** *Invest Ophthalmol Vis Sci*, **39**(12):2427-2442
- van Driel WD, van Leeuwen EJ, Von den Hoff JW, Maltha JC, Kuijpers-Jagtman AM (2000): **Time-dependent mechanical behaviour of the periodontal ligament.** *Proc Inst Mech Eng H*, **214**(5):497-504.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002): **Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.** *Genome Biol*, **3**(7):RESEARCH0034.
- Vandesompele J, Kubista M, Pfaffl MW (2009a): **Chapter 4: Reference Gene Validation Software for Improved Normalization:** Caister Academic Press.
- Vandesompele J, Kubista M, Pfaffl MW (2009b): **Chapter 5: Data Analysis Software:** Caister Academic Press.
- Verma MK, Yateesh AN, Smitha R, Neelima K, Pallavi PM, Reddy M, Poornima J, Oommen AM, Jagannath MR, Somesh BP (2014): **Integrated analysis of chronic lipotoxicity on muscle metabolism and stress and its reversal by antioxidants.** *Springerplus*, **3**:251
- Vianna Freitas Fachin E, Oliveira Filho E, Pinto Carvalho T (2007): **Caries Removal Based On Pulp Biology.** *Electronic Journal of Endodontics Rosario*, **1**(6):14.

- Virtej A, Loes S, Iden O, Bletsa A, Berggreen E (2013): **Vascular endothelial growth factors signalling in normal human dental pulp: a study of gene and protein expression.** *Eur J Oral Sci*, **121**(2):92-100
- Vogel C, Marcotte EM (2012): **Insights into the regulation of protein abundance from proteomic and transcriptomic analyses.** *Nat Rev Genet*, **13**(4):227-232.
- Von Bohl M, Maltha J, Von den Hoff H, Kuijpers-Jagtman AM (2004): **Changes in the periodontal ligament after experimental tooth movement using high and low continuous forces in beagle dogs.** *Angle Orthod*, **74**(1):16-25.
- von Bohl M, Ren Y, Fudalej PS, Kuijpers-Jagtman AM (2012): **Pulpal reactions to orthodontic force application in humans: a systematic review.** *J Endod*, **38**(11):1463-1469
- Von Bohl M, Ren Y, Kuijpers-Jagtman AM, Fudalej PS, Maltha JC (2016): **Age-related changes of dental pulp tissue after experimental tooth movement in rats.** *PeerJ*, **4**:e1625.
- Vongsavan N, Matthews B (1992): **The vascularity of dental pulp in cats.** *J Dent Res*, **71**(12):1913-1915.
- Vuolteenaho K, Koskinen A, Kukkonen M, Nieminen R, Paivarinta U, Moilanen T, Moilanen E (2009): **Leptin enhances synthesis of proinflammatory mediators in human osteoarthritic cartilage--mediator role of NO in leptin-induced PGE2, IL-6, and IL-8 production.** *Mediators Inflamm*, **2009**:345838.
- Wang GL, Jiang BH, Rue EA, Semenza GL (1995): **Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension.** *Proc Natl Acad Sci U S A*, **92**(12):5510-5514
- Wang H, Yin Y, Li W, Zhao X, Yu Y, Zhu J, Qin Z, Wang Q, Wang K, Lu W *et al* (2012): **Over-expression of PDGFR-beta promotes PDGF-induced proliferation, migration, and angiogenesis of EPCs through PI3K/Akt signaling pathway.** *PLoS One*, **7**(2):e30503.
- Wang L, Xiong M, Che D, Liu S, Hao C, Zheng X (2000): **The effect of hypoxia on expression of basic fibroblast growth factor in pulmonary vascular pericytes.** *J Tongji Med Univ*, **20**(4):265-267
- Wang WH, McNatt LG, Shepard AR, Jacobson N, Nishimura DY, Stone EM, Sheffield VC, Clark AF (2001): **Optimal procedure for extracting RNA from human ocular tissues and expression profiling of the congenital glaucoma gene FOXC1 using quantitative RT-PCR.** *Mol Vis*, **7**:89-94.
- Wang Y, Liu CL, Storey JD, Tibshirani RJ, Herschlag D, Brown PO (2002): **Precision and functional specificity in mRNA decay.** *Proc Natl Acad Sci U S A*, **99**(9):5860-5865
- Warrington JA, Nair A, Mahadevappa M, Tsyganskaya M (2000): **Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes.** *Physiol Genomics*, **2**(3):143-147
- Wehrle C, Van Slyke P, Dumont DJ (2009): **Angiopoietin-1-induced ubiquitylation of Tie2 by c-Cbl is required for internalization and degradation.** *Biochem J*, **423**(3):375-380
- Weis SM, Cheresh DA (2011): **alphaV integrins in angiogenesis and cancer.** *Cold Spring Harb Perspect Med*, **1**(1):a006478.

- Wiig H, Rubin K, Reed RK (2003): **New and active role of the interstitium in control of interstitial fluid pressure: potential therapeutic consequences.** *Acta Anaesthesiol Scand*, **47**(2):111-121.
- Willam C, Koehne P, Jurgensen JS, Grafe M, Wagner KD, Bachmann S, Frei U, Eckardt KU (2000): **Tie2 receptor expression is stimulated by hypoxia and proinflammatory cytokines in human endothelial cells.** *Circ Res*, **87**(5):370-377
- Willems E, Leyns L, Vandesompele J (2008): **Standardization of real-time PCR gene expression data from independent biological replicates.** *Anal Biochem*, **379**(1):127-129
- Winkler IG, Barbier V, Nowlan B, Jacobsen RN, Forristal CE, Patton JT, Magnani JL, Levesque JP (2012): **Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance.** *Nat Med*, **18**(11):1651-1657
- Wise SG, Waterhouse A, Michael P, Ng MK (2012): **Extracellular matrix molecules facilitating vascular biointegration.** *J Funct Biomater*, **3**(3):569-587
- www.string-db.org
- Yadav SS, Narayan G (2014): **Role of ROBO4 signalling in developmental and pathological angiogenesis.** *Biomed Res Int*, **2014**:683025
- Yamaguchi MK, K (2007): **The Effects of Orthodontic Mechanics on the Dental Pulp.** In: *Seminars in Orthodontics*. vol. 13: 272-280
- Yamanaka H, Obata K, Kobayashi K, Dai Y, Fukuoka T, Noguchi K (2007): **Activation of fibroblast growth factor receptor by axotomy, through downstream p38 in dorsal root ganglion, contributes to neuropathic pain.** *Neuroscience*, **150**(1):202-211
- Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J (2000): **Vascular-specific growth factors and blood vessel formation.** *Nature*, **407**(6801):242-248.
- Yano S, Nishioka Y, Goto H, Sone S (2003): **Molecular mechanisms of angiogenesis in non-small cell lung cancer, and therapeutics targeting related molecules.** *Cancer Sci*, **94**(6):479-485
- Yao VJ, Ozawa MG, Varner AS, Kasman IM, Chanthery YH, Pasqualini R, Arap W, McDonald DM (2006): **Antiangiogenic therapy decreases integrin expression in normalized tumor blood vessels.** *Cancer Res*, **66**(5):2639-2649
- Yeh HJ, Ruit KG, Wang YX, Parks WC, Snider WD, Deuel TF (1991): **PDGF A-chain gene is expressed by mammalian neurons during development and in maturity.** *Cell*, **64**(1):209-216.
- Yongchaitrakul T, Pavasant P (2007): **Transforming growth factor-beta1 up-regulates the expression of nerve growth factor through mitogen-activated protein kinase signaling pathways in dental pulp cells.** *Eur J Oral Sci*, **115**(1):57-63.
- Yoshida S, Ohshima H (1996): **Distribution and organization of peripheral capillaries in dental pulp and their relationship to odontoblasts.** *Anat Rec*, **245**(2):313-326.
- Yu C, Abbott PV (2007): **An overview of the dental pulp: its functions and responses to injury.** *Aust Dent J*, **52**(1 Suppl):S4-16.
- Yu EZ, Burba AE, Gerstein M (2007): **PARE: a tool for comparing protein abundance and mRNA expression data.** *BMC Bioinformatics*, **8**:309

- Yuan G, Nanduri J, Khan S, Semenza GL, Prabhakar NR (2008): **Induction of HIF-1alpha expression by intermittent hypoxia: involvement of NADPH oxidase, Ca²⁺ signaling, prolyl hydroxylases, and mTOR.** *J Cell Physiol*, **217**(3):674-685.
- Zainal Ariffin SH, Yamamoto Z, Zainol Abidin IZ, Megat Abdul Wahab R, Zainal Ariffin Z (2011): **Cellular and molecular changes in orthodontic tooth movement.** *ScientificWorldJournal*, **11**:1788-1803.
- Zarzynska JM (2014): **Two faces of TGF-beta1 in breast cancer.** *Mediators Inflamm*, **2014**:141747
- Zengo AN, Bassett CA, Pawluk RJ, Prountzos G (1974): **In vivo bioelectric potentials in the dentoalveolar complex.** *Am J Orthod*, **66**(2):130-139.
- Zhang H, Cheng X, Richter M, Greene MI (2006): **A sensitive and high-throughput assay to detect low-abundance proteins in serum.** *Nat Med*, **12**(4):473-477
- Zhang J, Byrne CD (1999): **Differential priming of RNA templates during cDNA synthesis markedly affects both accuracy and reproducibility of quantitative competitive reverse-transcriptase PCR.** *Biochem J*, **337** (Pt 2):231-241
- Zhang JQ, Nagata K, Iijima T (1998): **Scanning electron microscopy and immunohistochemical observations of the vascular nerve plexuses in the dental pulp of rat incisor.** *Anat Rec*, **251**(2):214-220.
- Zhang X, Reinsvold P, Thibeault DW, Ekekezie, II, Rezaiekhalthigh M, Mabry SM, Buch S, Truog WE (2005): **Responses of pulmonary platelet-derived growth factor peptides and receptors to hyperoxia and nitric oxide in piglet lungs.** *Pediatr Res*, **57**(4):523-529
- Zhang Y, Wang L, Dey S, Alnaeeli M, Suresh S, Rogers H, Teng R, Noguchi CT (2014): **Erythropoietin action in stress response, tissue maintenance and metabolism.** *Int J Mol Sci*, **15**(6):10296-10333
- Zhong H, Simons JW (1999): **Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia.** *Biochem Biophys Res Commun*, **259**(3):523-526.
- Zhong S, Zhang S, Bair E, Nares S, Khan AA (2012): **Differential expression of microRNAs in normal and inflamed human pulps.** *J Endod*, **38**(6):746-752.
- Zhou WC, Zhang QB, Qiao L (2014): **Pathogenesis of liver cirrhosis.** *World J Gastroenterol*, **20**(23):7312-7324
- Zhu Q, Safavi KE, Spangberg LS (1998): **Integrin expression in human dental pulp cells and their role in cell attachment on extracellular matrix proteins.** *J Endod*, **24**(10):641-644.
- Zittermann SI, Issekutz AC (2006): **Basic fibroblast growth factor (bFGF, FGF-2) potentiates leukocyte recruitment to inflammation by enhancing endothelial adhesion molecule expression.** *Am J Pathol*, **168**(3):835-846
- Zulueta JJ, Sawhney R, Kayyali U, Fogel M, Donaldson C, Huang H, Lanzillo JJ, Hassoun PM (2002): **Modulation of inducible nitric oxide synthase by hypoxia in pulmonary artery endothelial cells.** *Am J Respir Cell Mol Biol*, **26**(1):22-30.
- Zund G, Nelson DP, Neufeld EJ, Dzus AL, Bischoff J, Mayer JE, Colgan SP (1996): **Hypoxia enhances stimulus-dependent induction of E-selectin on aortic endothelial cells.** *Proc Natl Acad Sci U S A*, **93**(14):7075-7080